ADVERTIMENT. La consulta d'aquesta tesi queda condicionada a l'acceptació de les següents condicions d'ús: La difusió d'aquesta tesi per mitjà del servei TDX (www.tesisenxarxa.net) ha estat autoritzada pels titulars dels drets de propietat intel·lectual únicament per a usos privats emmarcats en activitats d'investigació i docència. No s'autoritza la seva reproducció amb finalitats de lucre ni la seva difusió i posada a disposició des d'un lloc aliè al servei TDX. No s'autoritza la presentació del seu contingut en una finestra o marc aliè a TDX (framing). Aquesta reserva de drets afecta tant al resum de presentació de la tesi com als seus continguts. En la utilització o cita de parts de la tesi és obligat indicar el nom de la persona autora.

ADVERTENCIA. La consulta de esta tesis queda condicionada a la aceptación de las siguientes condiciones de uso: La difusión de esta tesis por medio del servicio TDR (www.tesisenred.net) ha sido autorizada por los titulares de los derechos de propiedad intelectual únicamente para usos privados enmarcados en actividades de investigación y docencia. No se autoriza su reproducción con finalidades de lucro ni su difusión y puesta a disposición desde un sitio ajeno al servicio TDR. No se autoriza la presentación de su contenido en una ventana o marco ajeno a TDR (framing). Esta reserva de derechos afecta tanto al resumen de presentación de la tesis como a sus contenidos. En la utilización o cita de partes de la tesis es obligado indicar el nombre de la persona autora.

WARNING. On having consulted this thesis you're accepting the following use conditions: Spreading this thesis by the TDX (www.tesisenxarxa.net) service has been authorized by the titular of the intellectual property rights only for private uses placed in investigation and teaching activities. Reproduction with lucrative aims is not authorized neither its spreading and availability from a site foreign to the TDX service. Introducing its content in a window or frame foreign to the TDX service is not authorized (framing). This rights affect to the presentation summary of the thesis as well as to its contents. In the using or citation of parts of the thesis it's obliged to indicate the name of the author

UNIVERSITAT POLITÈCNICA DE CATALUNYA
BARCELONATECH

Departament d'Enginyeria Química

PhD Thesis

Investigation of the antioxidant properties of five aromatic plants in model food systems

Nurul Aini Binti Mohd Azman

PhD Supervisor:

Dra. María Pilar Almajano Pablos

Program of Chemical Process Engineering
Department of Chemical Engineering
Universitat Politècnica de Catalunya
Barcelona, September 2015

ACKNOWLEDGEMENT

Special appreciation goes to my PhD director, Professor Dra Maria Pilar Almajano Pablos, for all her constant supports, thrusting and helping through ups and downs during this study. Her invaluable, sincere and priceless help, comments and suggestions have contributed to the success of this research. She has never failed to guide me, believed in me to go through all process in getting PhD. Thank you very much for all dedicated hours and hours discussing especially inspiration and motivation advice to get all research done (and be my shoulder to cry ☺).

No forgotten members of CSIC, Dr. Luis Julia, Dr. Luis Fajari and Venci for all collaboration and guided me to plan and perform the experimental in CSIC.

I thank as well all my colleagues in the Department of Chemical Engineering in ESTEIB, UPC; Monika, Gaby, Fran, Julia, Sara, Anita, Xisca and others thank you so much for all of your help and kindness. My acknowledgement also goes to all the technicians and office staffs of School of Chemical Engineering for their cooperations.

I'd like to express my sincere gratitude to my Malaysian@Spain brothers and sisters here in Barcelona (Zul J, Farah, Zufar, Binyamin, Raja, Nisak, Zainul, Zul Abdullah and vilanovians, Anuar and terassians). Secondly, my love and appreciation goes two kind hearted women I know in Spain; Kak Akma and Kak Sobiah, for your supports, help and trust unconditionally.

I also would like to thank Ministry of Higher Education Universiti Malaysia Pahang for scholarship opportunity to pursue my study in Spain.

And last, but not least, my eternal gratitude goes to love of my life, my son Luqman Alhakim that very patient accompany me during my study.. He brings the joys in my life, smiles whenever I cried, bring hope whenever I demotivate and my little princess Aesyah Imanina, thank you God for the two precious gift in my life. Last but not least, thank you my husband, Shafik Husni for teach me many things in my life.

May only God return all your deeds and kindness…

THESIS OUTLINE

The thesis, entitled Investigation of the Antioxidant Properties of Five Aromatic Plants in Model Food Systems, was submitted for PhD approval to the chemical engineering department at Technical University of Catalonia on 20 May 2015.

The thesis is divided into five main chapters. The main content of each corresponding chapter will be detailed as follows.

Chapter 1 (Introduction) consists of four subchapters, i.e., 1.1, 1.2, 1.3 and 1.4. In Chapter 1, an overview is given of the state of the art in the field of free radicals and antioxidant mechanisms relevant to human health. The problem statement of current antioxidant study is also presented. Finally, the main goal and objectives of the thesis are established and elaborated upon in the final subtopic.

In Chapter 2, an explanation with comprehensive citation of the natural sources of antioxidant is described in the first subtopic, 2.1. Special emphasis is paid to the pharmacological description of the five research plants including white tea, yellow gentian, field binweeds, silver birch and common bearberry in subchapter 2.2. The findings of the biological benefits of those five medicinal plants for human benefit are highlighted in the corresponding chapter. Furthermore, the research on recent technology is clarified in the following subchapter, 2.3. This compromises the latest technology of antioxidant activity evaluation in food models such as emulsion, meat and active packaging.

Chapter 3 provides the main content of this thesis, describing five accepted journal articles published in various journals. The 3.1 subchapter describes the new radical scavenging method measured by electron paramagnetic resonance (EPR). This newly developed method generated by Fenton's reaction measures white tea methanol extract and its catechins to determine the scavenging activity toward the methoxy radical generated by Fenton's reaction. This paper was accepted in the Journal of Agriculture and Food Chemistry on 2 June 2014. The second accepted paper was established in the Journal of The Food Science and Agriculture on 19 August 2014 (subchapter 3.2). This study reflects

the various concentrations of yellow gentian root extract tested to delay the oxidation rate of meat patties. The following subchapter (3.3) demonstrates the third accepted paper published in MDPI Antioxidant on 12 June 2014. Yellow gentian root antioxidant activity potential in oil–water emulsion and identification of relevant compounds toward antioxidant effects are the main concentrations of this study. Meanwhile, the implementation of field bindweed in food models and formulating the extract in film packaging is investigated in subtopic 3.4. The study determines the potential of field bindweed antioxidant activity in muscle foods and coated the lyophilise plant in active films to inhibit the oxidation process. The research was published in MDPI Antioxidant on 10 March 2015. Finally, the effect of different polarities of solvents on the extraction of silver birch and field binweed is demonstrated in subchapter 3.5. This section exhibits the different yields using various polarities of solvents measured by antioxidant in-vitro assays including Trolox equivalent antioxidant capacity (TEAC), Oxygen radical absorbance capacity (ORAC), Ferric Reducing Ability Of Plasma (FRAP) and DPPH assay. The paper was accepted in a special edition of the International Journal of Biological, Food, Veterinary and Agricultural Engineering on 10 November 2013.

Chapter 4 describes the study of silver birch and common bearberry in various antioxidant activity assays. The first subchapter, 4.1, observes the results of common bearberry leaves on oil–water emulsion and active packaging as a potent natural antioxidant in food models. The second subchapter, 4.2, shows the study of silver birch extract on muscle food and active packaging film. This study also is extended to identify the phenolic constituents that may be relevant regarding the antioxidant activity of silver birch. Thus, these two subchapters were submitted to Meat Science and Journal of the American Oil Chemists' Society for review.

Chapter 5 presents the conclusion and the recommendations of the respective research. The general conclusion of the research is elaborated further in subchapter 5.1, giving an overview of the main achievements of this thesis. Future recommendations on topics for future research as derived from this research are clarified in subchapter 5.2. Finally, the list of publications and conference involvement is detailed in Chapter 6.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION Page

1.1 Overview of Free radical and Antioxidant in Human health
- Free Radicals and Oxidative stress 3
- Antioxidant mechanism and its important to human health 5

1.2 Problem Statement
- Problems related Antioxidants 7

1.3 Objectives 9

1.4 The Scope of the study 11

References 13

CHAPTER 2: LITERATURE REVIEW

2.1 Natural Antioxidant
- Antioxidant-related compounds 19
- Plant as sources of natural antioxidants 24

2.2 Research Plant
- White tea leaves (*Camellia sinensis*) 28
- Yellow Gentian root (*Gentiana Lutea*) 30
- Field bindweed leaves (*Convolvulus Arvensis*) 31
- Silver Brich leaves (*Betula Pendula Roth.*) 32

- Common Bearberry leaves (*Arctostaphylos uva-ursi L. Sprengel*) 33

2.3 Antioxidant effect in food models

- Oil-in-water (O/W) emulsions 35
- Meat 36
- Active packaging film 37

References 39

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Radical Scavenging of White Tea and of its Flavonoid Constituents by EPR Spectroscopy 53

3.2 Use of lyophilised and powdered *Gentiana lutea* root in fresh beef patties stored under different atmospheres 73

3.3 Screening of Antioxidant Activity of *Gentian Lutea* Root and Its Application in Oil-in-Water Emulsions 93

3.4 The Effect of *Convolvulus Arvensis* Dried Extract as Potential Antioxidant in Food Models 115

3.5 Solvent effect on antioxidant activity and total phenolic Content of *Betula Alba* and *Convolvulus Arvensis* 135

3.6 A Study of the Properties of Bearberry Leaf Extract as Natural Antioxidant in Model Foods s 153

3.7 Evaluation of the antioxidant activity of *Betula Pendula Roth*. leaf extract (BP) and its effects on model foods. 169

CHAPTER 4: CONCLUSIONS AND RECOMENDATION

4.1	Overall conclusions	193
4.2	Recommendation for Future Research	195

ANNEX

6.1	Journal publication	199
6.2	Journal under review	200
6.3	Conferences	200

CHAPTER 1

CHAPTER 1: INTRODUCTION

1.1 Overview of Free Radicals and Antioxidants in Human Health

Free Radicals and Oxidative Stress

The fields of free radicals and antioxidants, or ‘redox biology’, are the basic fundamentals to aerobic life, which are closely related to human health. Free radical and reactive oxygen species (ROS) and reactive nitrogen species (RNS) are constantly formed in the human body[1] through the production of normal cellular metabolism[2]. Free radicals are atoms, molecules or ions with unpaired electrons that are highly unstable and active towards chemical reactions with other molecules. Free radical formation occurs continually in the cells by enzymatic and non-enzymatic reactions. Enzymatic reactions are involved in the human respiratory chain, in phagocytosis and in prostaglandin synthesis, and in the cytochrome P-450 system[3] while non-enzymatic reactions involve organic compounds initiated by ionizing reactions[4]. Despite the free radicals derived from the normal metabolic process in the human body, environmental factors also generate oxidative stress that influences human health and disease. It is known as environmental oxidative stress, and the factors are pollution, UV radiation, infrared radiation, ionizing radiation and nutrition[5].

Free radicals are derived from three elements: oxygen, nitrogen and sulfur, creating ROS (reactive oxygen species), RNS (reactive nitrogen species) and RSS (reactive sulfur species). In normal physiological conditions, only 2% of the oxygen consumed by the body is converted through mitochondrial respiration and phagocytosis[6]. However, ROS formation is increased when our bodies are exposed to the environmental oxidative stress conditions which later convert to strongly oxidizing radicals. ROS are very reactive and subsequently react with other molecules to form secondary radicals such as hydroxyl radicals ($^{\bullet}OH$), alkoxyl radicals ($RO^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$) and singlet oxygen ($^{1}O_2$). Some radical species are converted to molecular oxidants like hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hypochlorous acid (HOCl), and these molecular species may act

as a source of ROS[7]. RNS are derived from NO by reacting with O_2 and forming $ONOO^-$, while RSS are easily formed by the reaction with thiols[8,9]. Figure 1 shows the reactions leading to the formation of ROS.

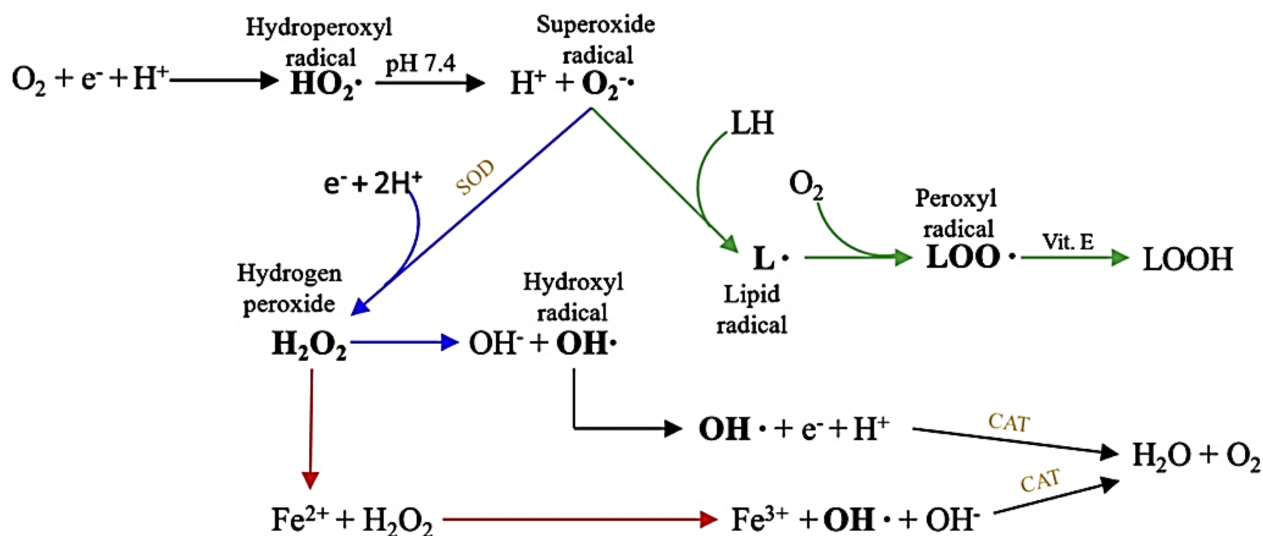


Figure 1: Production of ROS via different mechanism routes. The blue arrow represents the Haber-Weiss reaction and the red arrows represent the Fenton reaction. The bold letter represents the radical or molecule with the same behaviour (H_2O_2). The green arrow represents lipid oxidation (Source: Carocho et al., 2013[9])

ROS (with RSS and RNS) play dual roles as deleterious and beneficial species, and they both can be harmful or beneficial to living system. The beneficial effect of ROS occurs at low concentration and involves physiological roles in cellular responses to noxia—for example in defence against infectious agents and in the function of a number of cellular signalling systems[2]. In contrast, at high concentrations, ROS can be important mediators of damage to cell structures, including lipids, membranes, proteins and nucleic acids known as oxidative stress[10]. Oxidative stress occurs as a result of an imbalance between free radical production and antioxidant defence[4]. Short-term oxidative stress occurs in tissues injured by trauma, infection, heat injury, hypertoxia, toxins and excessive exercise[4]. Oxidative damage accumulates during the life cycle due to ROS formation, which has been proposed

to play a key role in the development of age-dependent diseases such as cancer, arteriosclerosis, arthritis, neurodegenerative disorders and other conditions. ROS have been implicated in the induction and complications of diabetes mellitus, cardiovascular diseases and eye diseases[11]. Thus, the harmful effects of ROS are balanced by the antioxidant action of non-enzymatic antioxidants in addition to antioxidant enzymes[12].

Antioxidant Mechanism and its Importance to Human Health

Antioxidants are stable molecules which donate an electron to a rampaging free radical and neutralize it, thus reducing the arisen of oxidative stress. Antioxidants act as radical scavengers, hydrogen or electron donors, peroxide decomposers, singlet oxygen quenchers, enzyme inhibitors, synergist and metal chelating agents[13]. Antioxidants are compounds or systems that delay autoxidation by inhibiting formation of free radicals or by interrupting propagation of the free radical chain by one or several mechanisms[14]. The summary of the five mechanisms involved in antioxidant reactions are (1) scavenging species that initiate peroxidation, (2) chelating metal ions that are unable to generate reactive species or decompose lipid peroxides, (3) quenching $^{\bullet}O_2^-$ preventing formation of peroxides, (4) breaking the autoxidative chain reaction or (5) reducing localized O_2 concentrations[15]. It is reported that antioxidants can execute protective roles against free radicals by a variety of different mechanisms including the catalytic systems to neutralize or divert ROS (shown in Figure 1); binding or inactivation of metal ions prevents generation of ROS by Haber-Weiss reaction; and suicidal and chain breaking antioxidants scavenge and destroy ROS or absorb energy, electrons and quenching of ROS[16].

Currently, demand for intake of antioxidant food or dietary antioxidants in the market has increased because they are believed to keep the body healthy and free from diseases. Furthermore, the potential beneficial effects of food containing antioxidants protecting against disease have been well established[16]. Therefore, certain nutrients and dietary components with antioxidant properties are important to defend against oxidative stress and damage induced by free radicals and protect against oxidative stress injury of the body. Low molecular weight antioxidants can safely interact with free radicals and terminate the chain reaction before the radical starts to cause damage. Some antioxidants are produced normally in the body such as glutathione, ubiquinol and uric acid[17]. However,

micronutrient antioxidants such as vitamin E (α-tocopherol), vitamin C (ascorbic acid) and B-carotene cannot be produce naturally in the body, thus they must be supplied in the diet.

Food consumption is a major source of exogenous antioxidants and provides more than 25,000 bioactive food constituents as nutrients in the daily typical human diet[16]. These abundant sources of nutrients may modify a multitude of processes that are related to different diseases. Generally, antioxidants are abundant in many daily consume plants such as vegetables and fruits and are also found in grain cereals, peas, legumes and nuts. Currently, food containing antioxidants are available commercially. There are more than 3,100 antioxidants in various foods, beverages, spices, herbs and supplements that are regularly consumed by different cultures[18].

Various studies have been conducted that are related to the mechanism of antioxidants in the human body, free radicals, oxidative stress and antioxidant activity of food. They have shown the prominent beneficial role of an antioxidant and its specific role against different diseases individually. There are a number of epidemiological studies that have shown an inverse correlation between the levels of established antioxidants/phytonutrients present in tissue/blood samples and occurrences of cardiovascular disease, cancer or mortality due to these diseases. Antioxidant-based drugs/formulations for prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease (AD), Parkinson's disease, cancer, etc. have appeared over the past three decades[19]. Since several plant products are rich in antioxidants and micronutrients, it is likely that dietary antioxidant supplementation protects against the oxidative stress that causes disease development[7]. The most recent report highlights the disparity between the recommendation for consumption of five portions of fruit and vegetables per day and the actual estimated intake in the population[20]. Fruit juices and vegetable juices have been shown to be a rich source of bioaccessible antioxidants and have shown to lower markers of oxidative stress and inflammation in a cohort of type 2 diabetes sufferers[21,22]. Furthermore, a recent meta-analysis of epidemiological studies concluded that there was sufficient evidence to recommend an increased consumption of green leafy vegetables in order to prevent the development of type 2 diabetes mellitus with a relative risk of 0.86 (95% CI=0.77–0.97, P=0.01) for consumption of 1.35 portions per day

(highest) compared with 0.2 portions per day (lowest)[23]. Below the summary (Table 1) of the health effect associated with the intake of antioxidants reported by Rajendran et al. (2014) is reported [16].

Table 1 : Effects of the health associated with the intake of antioxidants**[16]**.

Antioxidant	**Effect of Health**	**Reference no.**
Vitamin C	Protects against cancers, protects from heart disease, improves the health of cartilage, joints and skin, maintains a healthy immune system, improvement in the antibody production, increases the absorption of nutrients, increases protection against H_2O_2-induced DNA strand breaks	24–30
Vitamin E	Prevents coronary heart disease, prevents the formation of blood clots, decreases incidence of breast and prostate cancers, protects the brain, reduces long-term risk of dementia, decreases risk of Parkinson’s disease	31–35
Polyphenol	Inhibits oxidation of LDL, inhibits platelet aggregation, improves endothelial dysfunction, lowers risk of myocardial infarction, Has anti-carcinogenic effect, prevents neurodegenerative diseases, protects against neurotoxic drugs, treats diabetes, prevents osteoporosis, inhibit non-heme iron absorption	36–45
Cu, Zn, Mn, Se and other carotenoids (lycopene)	Cofactors of antioxidant enzymes SOD-Cu/Zn, Mn-SOD and GSH-Pox, as well as other carotenoids (lycopene); protection against oxidation of lipids, LDL, proteins and DNA; Abduction and free radical scavenging	46

1.2 Problem Statement

Problems Related to Antioxidants

The most frequently used synthetic antioxidants to preserve food are butylated hydro xyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butyl hydroquinone (TBHQ). They have been utilized as food additives and preservatives

to overcome the oxidation of foods due to their oil and fat contents and their stabilised shelf life. Today, almost all processed foods have synthetic antioxidants incorporated, which are reported to be safe—although some studies indicate otherwise[9]. These synthetic antioxidants which are widely used in the food industry are cheap, efficient, pure, easily available and harmless, if used at concentrations permitted by legislation. On the other hand, a few studies have found that the chemical compounds used as ~~of~~ synthetic antioxidants may be contributors to many health problems such as cancer growth and formation of mutagens at high level of intake[9,47]. Due to these concerns, an alternative strategy of industries was developed in many studies that could replace the synthetic antioxidants or at least diminish their uses as food additives. One of the strategies is to formulate natural sources of antioxidants in food products. Thus, the market demand for natural antioxidants commonly found in plants has increased. Since natural antioxidants are not only used as food preservatives, it is also believed that they add value to food products because of their nutritional content and benefits. The formulation of natural antioxidants in foods provides health benefits for consumers such as reduction in the incidence of cardiovascular diseases and cancer[48].

1.3 Objectives

The main objective of this thesis was to determine the antioxidant potential of five herbal plants which are white tea leaves (*Camellia Sinensis*), yellow gentian root (*Gentiana Lutea*), field bindweed leaves (*Convolvulus Arvensis Linn*), silver birch leaves (*Betula Pendula Roth*.) and common bearberry leaves (*Arctostaphylos uva-ursi L. Sprengel*).

The determination of antioxidant effects for each plant was to fill a research gap in the literature. White tea is widely studied for its antioxidant activity; thus, our study aimed to develop a new radical scavenging method using the Fenton reaction and measure the scavenging activity of white tea and the catechins in the plant extract. This new scavenging method is then further used to determine the scavenging of radical by field bindweed leaves (*Convolvulus Arvensis Linn*), silver birch leaves (*Betula Pendula Roth*.) and common bearberry leaves (*Arctostaphylos uva-ursi L. Sprengel).*

Yellow gentian (*Gentiana Lutea*), field bindweed leaves (*Convolvulus Arvensis Linn*), silver birch leaves (*Betula Pendula Roth*.) and Common Bearberry leaves (*Arctostaphylos uva-ursi L. Sprengel)* were analysed for their phenolic content and antioxidant activity using various *in-vitro* assays.

The enzymatic analysis and identification of relevant compounds for antioxidant effects was determined using *Gentiana Lutea* root.

The concentration effects to inhibit lipid oxidation in food models were determined for Yellow Gentian (*Gentiana Lutea*), Field bindweed leaves (*Convolvulus Arvensis Linn*), silver brich leaves (*Betula Pendula Roth*.) and common bearberry leaves (*Arctostaphylos uva-ursi L. Sprengel*).

Finally, field bindweed leaves (*Convolvulus Arvensis Linn*), silver brich leaves (*Betula Pendula Roth*.) and common bearberry leaves (*Arctostaphylos uva-ursi L. Sprengel*) were further investigated by formulating the extracts into gelatine film as active film packaging for food products.

1.4 The Scope of the Study

The scopes of this study are described in 3 phases:

Phase 1: Developing new techniques measuring the scavenging activity of white tea (*Camellia Sinensis*) by using EPR (Electron Paramagnetic Resonance) analysis.

a. Study of radical scavenging activity of Fenton reaction (methoxy radical) with different concentrations tested by EPR analysis.

b. Development of new calibration curve using Ferulic acid as a water-soluble standard in Fenton reaction measured by EPR.

c. Determination of radical scavenging activity of *Camellia Sinensis* and various catechins in Fenton reaction measured by EPR.

d. Determination of radical scavenging activity of *Betula Pendula Roth* and *Convolvulus Arvensis Linn* in Fenton reaction measured by EPR

Phase 2: Evaluation of various activities of *Gentiana Lutea* root associated with their antioxidant effect and identification of active compound and application in food models.

a. Study on extraction yield and solvent effect of *Gentiana Lutea.*

b. Quantification of total phenolic content (TPC) and *in vitro* antioxidant activity analysis of *Gentiana Lutea.* The *in vitro* antioxidant assays are 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP).

c. Study of the concentration effect on lipid stability of *Gentiana Lutea* extract in meat and emulsion.

d. Determination of enzymatic activity of *Gentiana Lutea* extracts using XO superoxide assays.

e. Identification of relevant compound responsible for the antioxidant activity of *Gentiana Lutea* using ABTS$^{\bullet+}$ radical-post-column injection in HPLC.

Phase 3: Screening of *Betula Pendula Roth.*, *Convolvulus Arvensis* and *Arctostaphylos uva-ursi L. Sprengel* antioxidant activity using various assays and their application in food models.

a. Determination of the extraction yield and solvent effect of *Betula Pendula Roth, Convolvulus Arvensis* and *Arctostaphylos uva-ursi L. Sprengel.*

b. Quantification of TPC and *in vitro* antioxidant activity analysis of *Betula Pendula Roth., Convolvulus Arvensis* and *Arctostaphylos uva-ursi L. Sprengel.* The *in vitro* antioxidant assays are 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP).

c. Study of the concentration effect on lipid stability for *Betula Pendula Roth, Convolvulus Arvensis* and *Arctostaphylos uva-ursi L. Sprengel* plants extract in meat and emulsion.

d. Study of the antioxidant effect on lipid stability for *Betula Pendula Roth, Convolvulus Arvensis and Arctostaphylos uva-ursi L. Sprengel* extract formulated with gelatine film as active film packaging..

References

1. Halliwell B. Free radicals and antioxidants - Quo vadis? *Trends Pharmacol Sci*. 2011;32:125-130.

2. Poli G, Leonarduzzi G, Biasi F, Chiarpotto E. Oxidative stress and cell signalling. *Curr Med Chem*. 2004;11:1163-1182.

3. Liu T, Stern A, Roberts LJ. The isoprostanes: Novel prostanglandin-like products of the free radical catalyzed peroxidation of arachidonic acid. *J Biomed Sci*. 1999;6:226-235.

4. Lobo V, Patil A, Phatak A, Chandra N. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn Rev*. 2010;4:118.

5. Tilman G. *Oxidants and Antioxidant Defense Systems*. 2nd ed. Dusseldorf, Germany; 2005:20.

6. Winterbourn CC. Reconciling the chemistry and biology of reactive oxygen species. *Nat Chem Biol*. 2008;4:278-286.

7. Kunwar A, Priyadarsini K. Free radicals, oxidative stress and importance of antioxidants in human health. *J Med Allied Sci*. 2011;1:53-60.

8. Lü J, Lin PH, Yao Q, Chen C. Chemical and molecular mechanisms of antioxidants: experimental approaches and model systems. *J Cell Mod Med*. 2010;14:840-860.

9. Carocho M, Ferreira ICFR. A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives. *Food Chem Toxicol*. 2013;51:15-25.

10. Valko M, Izakovic M, Mazur M, Rhodes CJ, Telser J. Role of oxygen radicals in DNA damage and cancer incidence. *Mol Cell Biochem*. 2004;266:37-56.

11. Rao AL, Bharani M, Pallavi V. Role of antioxidants and free radicals in health and disease. *Adv Pharmacol Toxicol*. 2006;7:29-38.

12. Halliwell B. Antioxidants in human health and disease. *Annu Rev Nutr*. 1996;16:33-50.

13. Frie B SRAB. Antioxidant defences and lipid peroxidation in human blood plasma. *Proc Natl Acad Sci*. 1988;37:569-571.

14. Brewer MS. Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications. *Compr Rev Food Sci Food Saf*. 2011;10:221-247.

15. Nawar WF. *Lipids.* 3 rd. (In: Fennema O, Chemistry F, ed.). New York, USA: Marcel Dekker, Inc.; 1996:225-320.

16. Rajendran P, Nandakumar N, Rengarajan T, et al. Clinica Chimica Acta Antioxidants and human diseases. *Clin Chim Acta*. 2014;436:332-347.

17. Halliwell B. How to characterize an antioxidant- An update. *Biochem Soc Symp*. 1995;61:73-101.

18. Matsuzawa A, Ichijo H. Redox control of cell fate by MAP kinase: physiological roles of ASK1-MAP kinase pathway in stress signaling. *Biochim Biophys Acta Gen*. 2008;1780:1325-1336.

19. Devasagayam TP a, Tilak JC, Boloor KK, Sane KS, Ghaskadbi SS, Lele RD. Free radicals and antioxidants in human health: current status and future prospects. *J Assoc Physicians India*. 2004;52:794-804.

20. Bates B. National diet and nutrition survey: Headline results from year 1 of the rolling programme (2008/2009). *Food Standards Agency*. 2010.

21. Wootton-Beard PC, Ryan L. Improving public health?: The role of antioxidant-rich fruit and vegetable beverages. *Food Res Int*. 2011;44:3135-3148.

22. Asgard R, Rytter E, Basu S, Abramsson-Zetterberg L, Moller L, Vessby B. High intake of fruit and vegetables is related to low oxidative stress and inflammation in a group of patients with type 2 diabetes. *Scandanavian J Food Nutr*. 2007;51:149-158.

23. Carter P, Gray LJ, Troughton J, Khunti K, Avies MJ. Fruit and vegetable intake and incidence of type 2 diabetes mellitus: Systematic review and metaanalysis. *Br Med Journal,*. 2010;341:4229.

24. Riso P, Martini D, Moller P. DNA damage and repair activity after broccoli intake in young healthy smokers. *Mutagenesis*. 2010:1-8.

25. Barry I. Vitamin C: friends or foe? *Nat Rev Cancer*. 2008;8:830.

26. Liu L, Zhao SP, Gao M, Zhou QCZ, Li YL, Xia B. Vitamin C preserves endothelial function in patients with coronary heart disease after a high-fat meal. *Clin Cardiol*. 2002;25:219-224.

27. Wang Y, Hodge AM, Wluka AE. Effect of antioxidante on knee cartilage and bone in healthy, middle-aged subjects: a cross-sectional study. *Arthritis Res Ther*. 2007;9:1-9.

28. Wintergerst ES, Maggini S, Homig DH. Immune-enhancing role of vitamin C and zinc and effect on clinical conditions. *Ann Nutr Metab*. 2006;50:85-94.

29. Woo A, Kim JH, Jeong YJ. Vitamin C acts indirectly to modulate isotype switching in mouse B cells. *Anat Cell Biol*. 2010;43:25-35.

30. Thankachan P, Walczyk T, Muthayya S, Kurpad A V., F. HR. Iron absorption in young Indian women: the interaction of iron status with the influence of tea and ascorbic acid. *Am J Clin Nutr*. 2008;87:881-886.

31. Pryor WL. Vitamin E and heart disease: basic science to clinical intervention trials. *Free Radic Biol Med*. 2000;28:141-161.

32. Traber MG, Frei B, Beckman JS. Vitamin E revisited: do new data validate for chronic disease prevention? *Curr Opin Lipidol*. 2008;19:30-38.

33. Weinstein SJ, Wright ME, Lawson KA. Serum and dietary vitamin E in relation to prostate cancer risk. *Prev, Cancer Epidemiol Biomarkers*. 2007;16:1253-1258.

34. Muller DPR. Vitamin E and neurological functions. *Mol Nutr Food Res*. 2010;54:1-9.

35. Devore EE, Grodstein F, van Rooij FJ. Dietary antioxidant and lonterm risk of dementia. *Arch Neurol*. 2010;67:819-825.

36. Hurrell RF, Reddy M, Cook JD. Inhibition of non-haem iron absorption in man by polyphenolic-containing beverages. *Br J Nutr*. 1999;81:289-295.

37. Manach C, Mazur A, Scalbert A. Polyphenols and prevention of cardiovascular diseases. *Curr Opin Lipidol*. 2005;16:77-84.

38. Russo P, Tedesco I, Russo M, Russo GL, Venezia A, Cicala C. Effects of de-alcoholated red wine and its phenolic fractions on platelet aggregation. *Nutr Metab Cardiovasc*. 2001;11:25-29.

39. Schachinger V, Britten MB, Zeiher AM. Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease. *Circulation*. 2000;101:1899-1906.

40. Corder R, Mullen W, Khan NQ. Oenology: red wine procyanidins and vascular health. *Nature*. 2006:444:566.

41. Yang CS, Landau JM, Huang MT, Newmark HL. Inhibition of carcinogenesis by dietary polyphenolic compounds. *Ann Rev Nutr*. 2001;21:381-406.

42. Halliwell B. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. *Drugs Aging*. 2001;18:685-716.

43. Pan T, Jankovic J, Le W. Potential therapeutic properties of green tea polyphenols in Parkinson's disease. *Drugs Aging*. 2003;20:711-721.

44. Zunino SJ, Storms DH, Stephensen CB. Diets rich in polyphenols and vitamin A inhibit the development of Type I autoimmune diabetes in nonobese diabetic mice. *J Nutr*. 2007;137:1216-1221.

45. Atmaca A, Kleerekoper M, Bayraktar M, Kucuk O. Soy isoflavones in the management of postmenopausal osteoporosis. Menopause. 2008;15:748-757.

46. Visioli F, Grande S, Bogani P, Galli C. The role of antioxidants in the mediterranean diets: focus on cancer. *Eur J Cancer Prev*. 2004;13:337-343.

47. Capitani CD, Carvalho ACL, Rivelli DP, Barros SBM, Castro I a. Evaluation of natural and synthetic compounds according to their antioxidant activity using a multivariate approach. *Eur J Lipid Sci Technol*. 2009;111:1090-1099.

48. Pereira ALF, Vidal TF, Teixeira MC, et al. Antioxidant effect of mango seed extract and butylated hydroxytoluene in bologna-type mortadella during storage. *Ciência e Tecnol Aliment*. 2011;31:135-140.

CHAPTER 2

CHAPTER 2: LITERATURE REVIEW

2.1 Natural Antioxidant

Antioxidant-related compounds

The effectiveness of antioxidant compounds can be defined in various ways: they prevent free radical oxidation reactions (preventive oxidants) by inhibiting the formation of free lipid radicals; they interrupt the propagation of the autoxidation chain reaction (chain-breaking antioxidants); they act as singlet oxygen quenchers; they create synergism with other antioxidants; they reduce the agents that convert hydroperoxides into stable compounds; they act as metal chelators that convert metal pro-oxidants (iron and copper derivatives) into stable products; and finally, they act as inhibitors of pro-oxidative enzymes (lipoxyigenases)[1–5]. Carocho et al. (2013) have illustrated that the human antioxidant system is divided into two major groups: enzymatic antioxidants and non-enzymatic antioxidants, as shown in Figure 1[6] (below).

The enzymatic antioxidants' action is divided into primary and secondary enzymatic defences and can be categorised according to three important enzymes that prevent the formation of or neutralise free radicals: glutathione peroxidase, catalase and superoxide dismutase. Whereas glutathione peroxidase donates two electrons to reduce peroxides by forming selenoles and also eliminates peroxides as a potential substrate for the Fenton reaction, catalase converts hydrogen peroxide into water and molecular oxygen, while superoxide dismutase converts superoxide anions into hydrogen peroxide as a substrate for catalase[10]. The secondary enzymatic defence involves glutathione reductase and glucose-6-phosphate dehydrogenase. Glutathione reductase changes oxidized glutathione into its reduced form and recycles it to neutralise more free radicals. Glucose-6-phosphate regenerates NADPH (nicotinamide adenine dinucleotide phosphate) and creates a reducing environment[11,12].

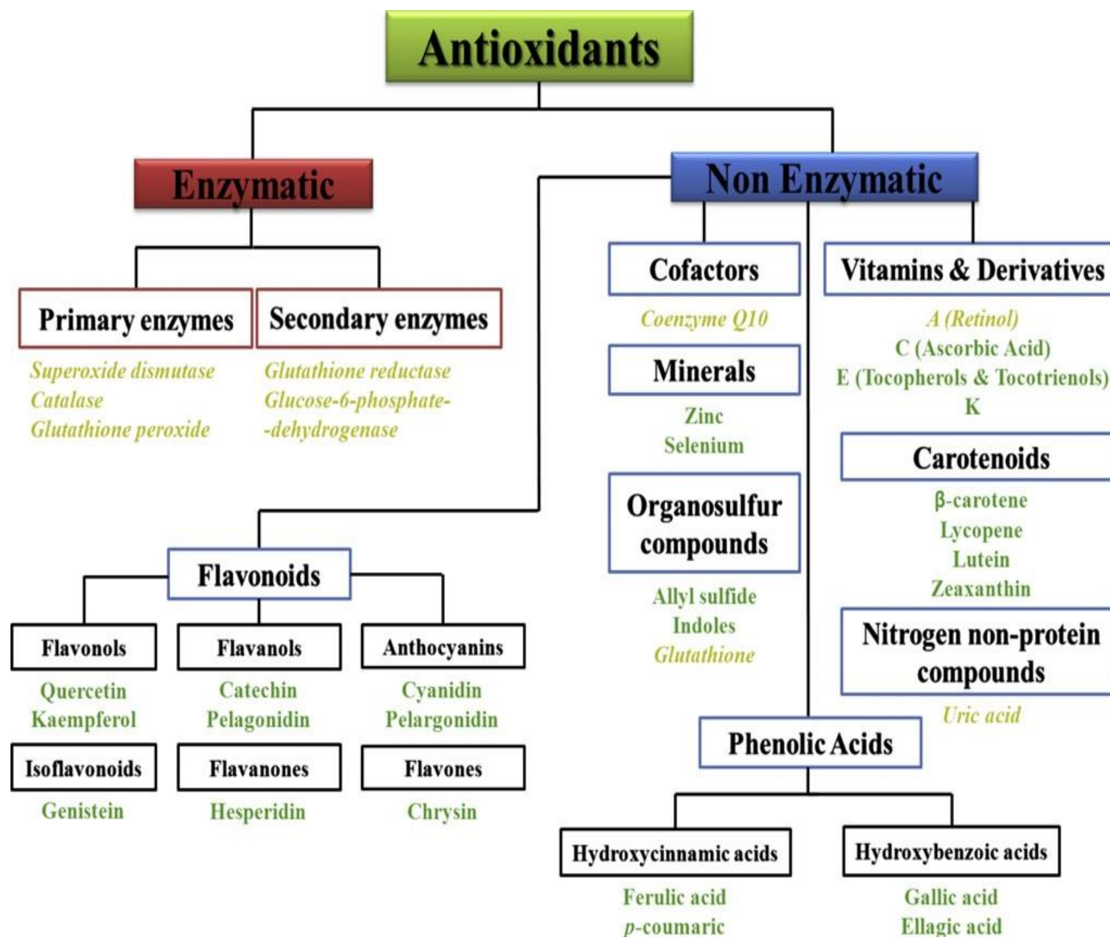


Figure 1: Classes of natural antioxidants. Green words represent exogenous antioxidants, and yellow ones represent endogenous antioxidants. This figure is reported from Carocho et al. (2013), who adapted it from Pietta (2000), Ratnam et al. (2006), and Godman et al. (2011)[7–9].

Vitamin A or retinol is a carotenoid derivative produced in the liver and results from the breakdown of b-carotene. Vitamin A is one of the non-enzymatic endogenous antioxidants, and there are about a dozen forms of the vitamin that can be isolated. It is known to have a beneficial effect on the skin, eyes and internal organs[6]. Coenzyme Q10 is present in all cells and membranes; it plays an important role in the respiratory chain and metabolism of other cells, and acts by preventing the formation of lipid peroxyl radicals. Turunen et al. (2004) reported that Coenzyme Q10 also regenerates vitamin E or causes the regeneration of vitamin E through ascorbate (vitamin C)[13]. Uric acid prevents lysis of erythrocytes by peroxidation and is a potent scavenger of singlet oxygen and hydroxyl radicals, as suggested by Kandar et al. (2006)[14]. Glutathione is an endogenous tripeptide

which protects the cells against free radicals either by donating a hydrogen atom or an electron[6]. It is also very important in the regeneration of other antioxidants like ascorbate (vitamin C)[15].

Vitamin C (ascorbic acid) compounds with antioxidant activity include L-ascorbic acid and L-dehydroascorbic acid, which are effective in scavenging the superoxide radical anion, hydrogen peroxide, hydroxyl radical, singlet oxygen and reactive nitrogen oxide[16]. Vitamin E (tocopherols) has the ability to halt lipid peroxidation by donating its phenolic hydrogen to peroxyl radicals, forming tocopheroxyl radicals that are unreactive and unable to continue the oxidative chain reaction[6]. Vitamin E is the only major lipid-soluble, chain-breaking antioxidant found in plasma, red cells and tissues, allowing it to protect the integrity of lipid structures, mainly membranes[17]. Vitamin K is a group of fat-soluble compounds, and the 1,4-naphthoquinone structure of this vitamin is what confers the antioxidant protective effect[6]. The structure of Vitamin E and C is illustrated in Figure 2[18].

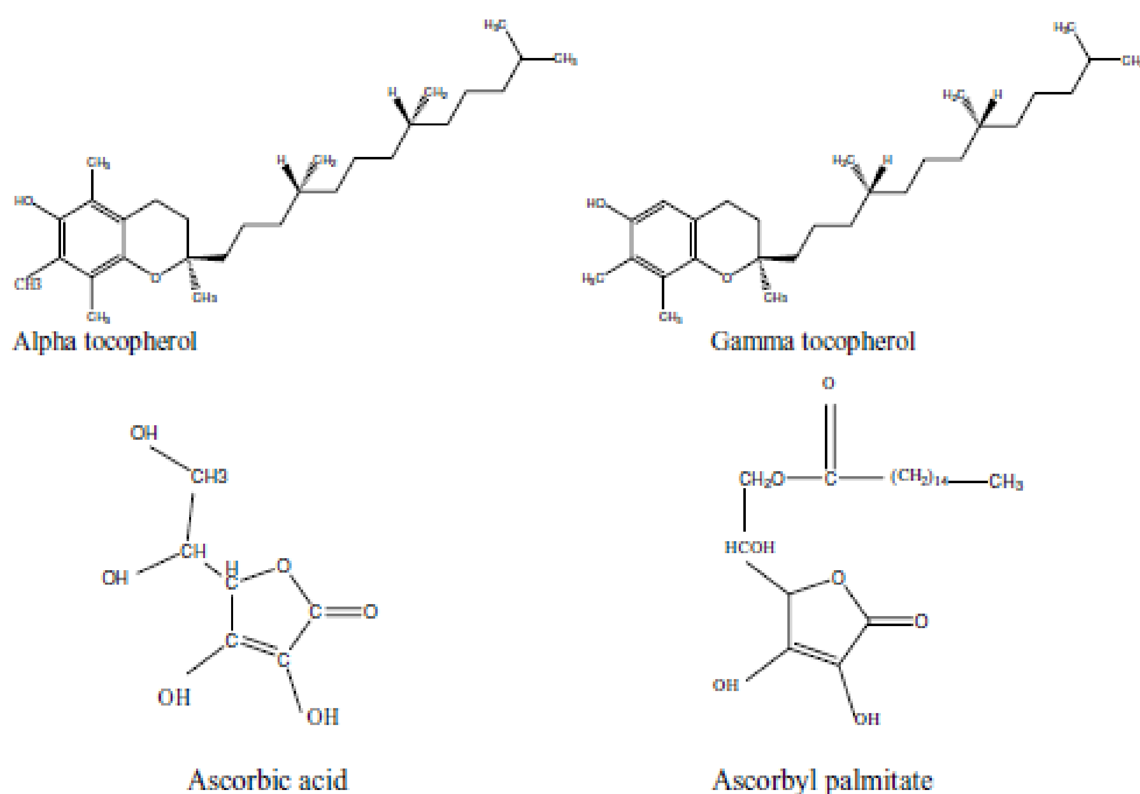


Figure 2: Vitamin C and Vitamin E group structures in natural antioxidants[18].

Carotenoids can be separated into two vast groups: the carotenoid hydrocarbons, known as carotenes, such as lycopene and b-carotene, and the oxygenated carotenoids, known as xanthophyls, such as zeaxanthin and lutein. The main antioxidant effect of carotenoids is due to their ability to quench singlet oxygen and to quench radical species. The mineral selenium is an indispensable part of most antioxidant enzymes (metalloenzymes, glutathione peroxidase, thioredoxin reductase), which would have no effect without it[19]. Meanwhile, zinc is also an inhibitor of NADPH oxidases and catalyzes the production of the superoxide radical using NADPH as an electron donor. With the addition of superoxide dismutase, zinc converts the superoxide radical into hydrogen peroxide[6].

Flavonoids are an antioxidant group of compounds composed of flavonols, flavanols, anthocyanins, isoflavonoids, flavanones and flavones. They are commonly found in plants and fruits. Antioxidant properties are conferred on flavonoids by the phenolic hydroxyl groups attached to ring structures. They can act as reducing agents, hydrogen donators, singlet oxygen quenchers, superoxide radical scavengers and even as metal chelators. They also activate antioxidant enzymes, reduce a-tocopherol radicals (tocopheroxyls), inhibit oxidases, mitigate nitrosative stress and increase levels of uric acid and low molecular weight molecules. Some of the most important flavonoids are catechin, catechin-gallate, quercetin and kaempferol, which are available commonly in herbs and fruits[20–23]. Brewer et al. (2011) illustrate the structure of the Flavonoids group found in plant extracts in Figure 3[18].

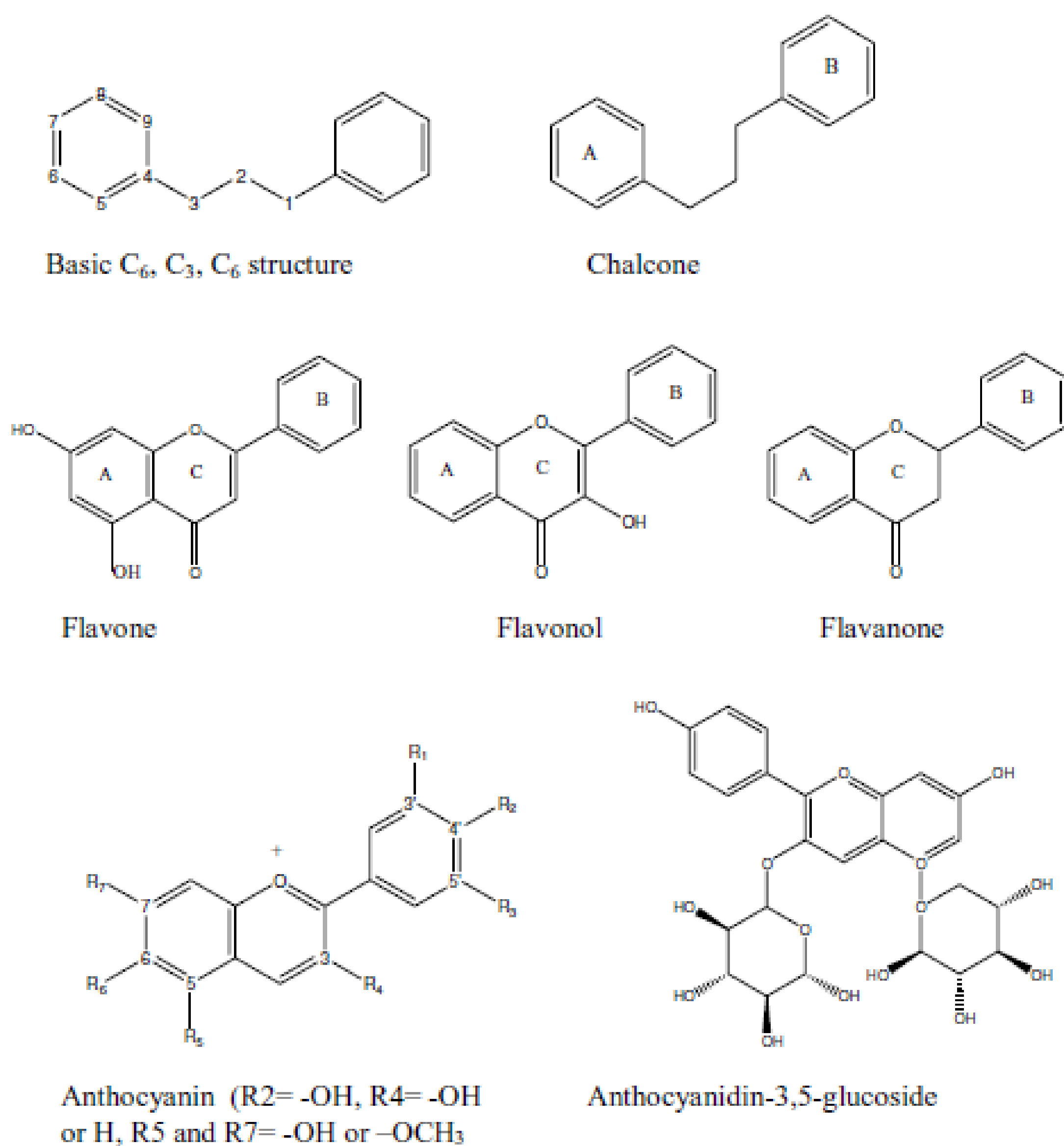


Figure 3: Flavonoid structure and flavonoids (flavanones: chalcone, flavone and flavanol; and flavans: anthocyanin and anthocyanidin-3,5-glycoside) found in plant extracts.

Phenolic acids are composed of hydroxycinnamic and hydroxybenzoic acids, commonly found in plant materials and sometimes present as esters and glycosides. They exhibit antioxidant activity as chelators and free radical scavengers with a special impact on hydroxyl and peroxyl radicals, superoxide anions and peroxynitrites[6]. One of the most studied and promising compounds in the hydroxybenzoic group is gallic acid, which is also the precursor of many tannins, while cinnamic acid is the precursor of all the

hydroxycinnamic acids[24,25]. The group structure of phenolic acids is shown in Figure 4 (source Brewer et al. (2011)[18]).

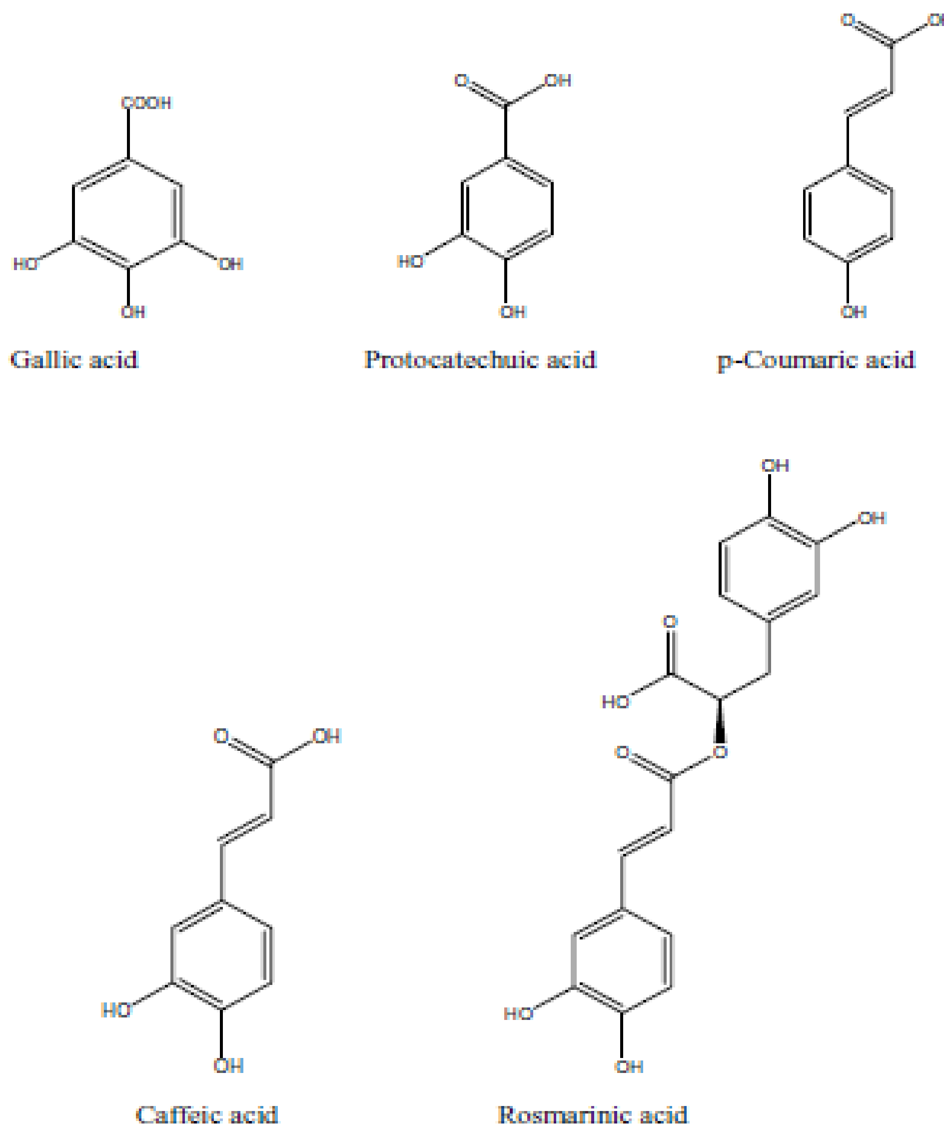


Figure 4: Structure of phenolic acid group found in natural antioxidants.

Plants as sources of natural antioxidants

Increasing demand has led to a rapid growth in the formulation of healthy, high quality and convenient foods, as well as natural oxidant-incorporated food and ready-to-eat food products[18]. Food quality is defined in terms of consumer acceptability: nutritional

value, taste, aroma and appearance characteristics. Many modern food ingredients contain unsaturated fatty acids that are quite susceptible to quality deterioration, especially under oxidative stress. For this reason, efforts to reduce oxidation have increased. Most often, the best strategy is the addition of antioxidants.

In nature, there are a wide variety of natural antioxidants which differ widely in their composition, physical properties, chemical properties, side actions and mechanisms. Antioxidant-related compounds can be grouped into a few categories: enzymes, and low and high molecular weight compounds such as phenolic acid, vitamins and minerals[26]. Most plant sources in which natural antioxidants can be obtained include culinary herbs, spices, fruits, vegetables and oilseed products[27]. For many years, research has been carried out to study plants as sources of potentially safe, natural antioxidants for the food industry; various compounds have been isolated, many of them being polyphenols[28,29].

Polyphenols or polyphenolic compounds, synthesized by plants, are polyhydroxylated phytochemicals and can be divided into 4 general groups: phenolic acids (gallic, protochatechuic, caffeic and rosmarinic acids), phenolic diterpenes (carnosol and carnosic acid), flavonoids (quercetin and catechin) and volatile oils (eugenol, carvacrol, thymol and menthol)[18]. Whereas phenolic acids act as antioxidants by trapping free radicals, flavonoids have the ability to scavenge free radicals and chelate metals as well[30]. Hence, due to their capacity to donate hydrogen atoms or electrons, phenolic acid and flavonoids act as antioxidants in blood and tissue in the body. The number of antioxidant flavonoids and polyphenols found in the average human diet (vegetables, tea, fruit and wine) is much higher than the amount of antioxidants such as vitamin E, vitamin C and carotenoids in the same foods. Phenolic compounds are the major constituents that contribute to the antioxidant properties of plant foods. Phenolic compounds such as phenolic acids, flavonoids, tocopherols and anthocyanins have radical scavenging properties and can be considered food antioxidants[31]. Many authors have demonstrated the ability of phenolic compounds to scavenge free radicals such as catechins[32,33], which are commonly found in tea. Therefore, the total amount of phenolic compounds is one of the most important factors affecting antioxidant activity. It has also been reported that most polyphenol compounds have anti-inflammatory, anti-fungal and antibacterial properties that

benefit human health[34]. Green tea extracts have the highest total phenolic content, 94% of which is in the form of flavonoids[35], while Oolong tea contains about 18% total phenolics and 4.4% flavonoids[18]. Theaflavins and thearubigins predominate in black tea, which also contains chlorogenic, caffeic, p-coumaric and quinic acids[36]

Besides phenolic acid and flavanoids, there are also many other compounds that have functional and nutritional value in edible plants, such as ascorbic acid[37], nitrogen compounds (amino acids, amines, alkaloids and chlorophyll derivatives)[38] and carotenoids[39]. These compounds play an important role not only as preservatives but also as nutrients and bioactive substances in the food industry. Ascorbic acid, also known as vitamin C, is a strong antioxidant found mainly in fresh fruits and vegetables. Ascorbic acid also exhibits synergic effects in its antioxidant activity, which prevents oxidation when combined with other antioxidant[40,41]. Chlorophylls and carotenoids are the most abundant plant pigments in nature and have antioxidant activity due to their singlet oxygen quenching properties[42]. Some carotenoids, such as α- and β-carotene, are precursors of vitamin A, which gives nutritional value to food[43]. Meanwhile, chlorophyll and its derivatives not only have antioxidant effects but may prevent certain types of cancers, aid in wound healing and reduce inflammation in some cases[44].

Many antioxidant constituents such as vitamins and carotenoids occur in the human dietary in fruits and vegetables. Fruits, vegetables, spices, herbs, cereals, grains, oilseeds, leguminous seeds, teas, coffee and cocoa are the major sources of plant-derived antioxidants such as phenolics and carotenoids, all of which have been extensively studied[27]. Wang et al. (2000) demonstrated the strong antioxidant activity found in fruits, and their research has been supported by many authors' studies on berries, cherries, kiwi, fruit juices and olives[45–47]. Several studies have established the antioxidant potential of a wide variety of vegetables including broccoli, spinach and oregano[48,49].

Meanwhile, medicinal plants continue to be a major source of drugs. Natural products, based on their therapeutic effects, are traditionally believed to have medicinal effects in all cultures[50]. Plants possess many potent bioactive compounds capable of preventing and treating most oxidative-related diseases and have often been used in folkloric medicine[51,52]. Lugasi et al. (1995) and Muchuweti et al. (2007) demonstrated that

several spices and herbs commonly used in foods for their flavour and in medicinal mixtures for their physiological effects also contain high concentrations of phenolic compounds with strong H-donating activity[53,54]. Meanwhile, Chen et al. (2007) demonstrated that various extracts of many members of the Labiatae (Lamiaceae) family (oregano, marjoram, savory, sage, rosemary, thyme and basil) contain high total phenol content, which is relevant to antioxidant activity[55]. This study was supported by Gallego et al. (2013), who showed that herbs such as thyme, rosemary and lavender possess antioxidant potential to inhibit lipid oxidation in an emulsion model[56]. However, Dorman et al. (2003) observed that the antioxidant characteristics are not entirely related to the total phenolic contents but are strongly dependent on rosmarinic acid, the major phenolic component present[57].

Natural antioxidant research has also focused on plant waste as a potential source of natural antioxidants. Oliveira et al. (2008) determined the presence of phenolic compounds in green walnut husks and also identified thirteen phenolic compounds: gallic acid, juglone, myricetin, chlorogenic acid, ellagic acid, protocatechuic acid, syringic acid, vanillic acid, catechin, epicatechin, ferulic acid, sinapic acid, chlirogenic acid and caffeic acid[58]. Lemon and orange peel exhibited antioxidant activity and scavenging for radicals activity using different solvent extracts[59].

Many parts of grape plants possess polyphenol content including flavonoids, phenolic acids, stilbenes, coumarins and lignoids. Phenolic compounds in grape seeds and skins include catechins, epicatechins, epicatechin-3-O-gallate, phenolic acids, caffeic acid, quercetin, myricetin, proanthocyanidins and resveratrol, which all exhibit strong antiradical activity[18]. The antioxidant activities of grape seed extract ranged from 66.4% to 81.4%, compared to vitamin E, which ranged from 90.3% to 94.7%. Grape seed extract has been shown to inhibit both lipid hydroperoxide and propanal formation in food models[60].

In comparison with the few effects of the antioxidants analysed above, it has been shown that there are many plant-derived compounds that have excellent antioxidant properties. Researchers have successfully shown that these compounds benefit human health. However, research work on antioxidants also suggests that the antioxidative activity of some promising plants is superior to synthetic antioxidants, making these plant sources

particularly attractive for commercial food processors due to consumer demand for natural ingredients.

2.2 Research plant

White tea leaves (*Camellia sinensis*)

Tea from the young buds and leaves of *Camellia sinensis* (L.) is the most widely consumed beverage in the world after water and is valued for its taste, aroma, health benefits and association with cultural practices[23]. The tea was originally planted in China but is now grown in tea plantations around the world to meet market demand. White, green, oolong, black and pu'erh teas are the major tea types sourced from leaves and buds of the tea plant. These are categorised based on variations in harvesting, processing and the associated degree of oxidation of polyphenols in the fresh tea leaves[61]. Of all these tea types, white tea is less well-known in western communities but is valued in Asia; the flavour is often preferred over green tea in Europe[62].

Recently, in the United States and Europe, white tea has received increasing attention, and research has been carried out into its nutritional quality. White tea is produced in very small quantities because the leaves are collected only at dawn during a few days in the spring when the buds are still closed. The traditional method used for white tea processing is to spread out the leaves to dry under the sun; during the lengthy drying process, in which the structure of the leaf cell is kept intact and not broken through any external physical interference such as curling or twisting, the tea becomes slightly "oxidized". This oxidation converts small amounts of catechins, which have been described as potent antioxidants, into theaflavins and thearubigins, which are responsible for the characteristic aroma and colour of black and oolong teas[63].

The difference between green and white tea production processes is that, in traditional Chinese green tea production, the mature tea leaves are withered, briefly pan-fried, rolled and dried. Some Chinese tea manufacturers steam rather than pan-fry their tea leaves, much like the Japanese style of green tea production[61,64]. Both tea heating methods

deactivate the polyphenol oxidase enzymes. Some published reports have shown that some white teas are also steamed during processing to deactivate enzymes. White teas have been reported to possess higher antielastase, anticollagenase, and antioxidative activity than certain green teas, and their suggested ability to promote strong and elastic skin and alleviate inflammation and rheumatoid arthritis has led to an increased interest in this tea type[65]. White tea lipolytic activity and its ability to inhibit adipogenesis have received particular attention, especially in developed countries battling with dramatic increases in obesity and obesity-related diseases[66].

The strong antioxidant activities of white tea are linked with a group of polyphenolic flavan-3-ol monomers and their gallate derivatives and catechins content[67]. The major catechins include (−)-epicatechin (EC), (−)- epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epigallocatechin-3-gallate (EGCG). These compounds are primarily responsible for many of the health protective properties associated with tea, including antioxidative[33], antiinflammatory[68], neuroprotective[69], anti-cancer[70] and antimicrobial[62] properties. In fact, recent studies have shown that white tea: exerts neuroprotection against hydrogen peroxide-induced toxicity in PC12 cells[71]; induces lipolytic activity and inhibits adipogenesis in human subcutaneous (pre)-adipocytes[66]; increases the antioxidant capacity of plasma and some organs in mice[72]; has potent antimutagenic activity in the Salmonella assay[73]; suppresses intestinal tumorigenesis in mice[74]; and inhibits pancreatic lipase activity in vitro[75]. Moreover, its ability to promote strong and elastic skin and alleviate inflammation and rheumatoid arthritis has led to an increased interest in this tea type[65]. Regarding the levels of catechins, total polyphenols and total antioxidant activity, white tea is, in general, not significantly different from green tea[76], even if some authors have found higher mean levels of some catechins and gallic acid in white tea compared to green teas[77]. Chinese white teas have also been reported to possess greater antimutagenic properties than premium green teas[73], as well as antioxidant effects, comparable to those of green teas, in body plasma and some organs[72]. White teas have also been found to contain higher amounts of caffeine than green teas[36], which, along with other methylxanthines such as TB (theobromine) and TP (theophylline), the amino acid theanine and free sugars, is a compound commonly found in tea. The numerous factors that affect the final tea product, which is consumed or analysed in a laboratory, include climate, soil,

plucking time, as well as processing and preparation methods[61]. The growing role of tea in people's diets in the United States over the past 2 decades[78] means that the inter-variation of beneficial compounds in green and white teas and the intra-variation within each tea type should be studied further.

Yellow Gentian root (*Gentiana Lutea*)

Gentiana lutea is a species belonging to the Gentianaceae family, a flowering plant comprising approximately 70–80 genera and 900–1,200 species. It is a perennial herb that grows commonly in mountainous areas of central and southern Europe and in western Asia[79,80]. It grows naturally on uncultivated ground in France, Spain and the Balkan mountains. *Gentiana lutea* root has been used as a medicinal plant with many pharmacological benefits and has been recognised for the bitter tasting of its root. The roots are cited for use in drug-making in many pharmacopoeias and are available commercially in the form of dried fermented rhizomes and roots[81]. Due to its bitter taste, the drug has been used as a stomachic or appetizer. Much work has been done to identify the active component in the *Gentiana Lutea* root. The bitter constituent is mainly due to the occurrence of secoiridoid-glycosides (e.g. swertiamarin (2), gentiopicroside (3), amarogentin (4) and andsweroside) in the plant[82], which have been shown to possess cholagogue, hepatoprotective and wound-healing effects in pharmacological studies[83]. Besides secoiridoids, other constituents that are relevant to the biological effects of gentian include iridoid loganic acid, which has an anti-inflammatory effect[84], xanthone glycosides and their derivative, and xanthones like gentisin and isogentisin, which have wide-ranging anti-inflammatory, anti-hepatotoxic, anti-tumour and anti-microbial effects[85]. Schmieder et al. (2007) revealed that the isogentisin compound in *Gentiana Lutea* root possesses protective effects against endothelial damage caused by cigarette smoking[86]. Aberham et al. (2011, 2007) evaluated the quality and efficiency of *Gentiana Lutea* by developing a new fully validated HPLC method for the simultaneous determination of bioactive compounds in the root extract[82,87].

Silver Birch leaves (*Betula Pendula Roth*.)

The genus *Betula* of the family Betulaceae, more commonly known as the birch tree, has a wide distribution in the northern hemisphere from Canada to Japan[88]. The birch tree has a long history of medicinal use in different countries and cultures and has been used to cure skin diseases such as eczema, infections, inflammations, rheumatism and urinary disorder[89]. Betula bud oil is also widely used in cosmetic products, mainly in hair products, as a tonic and antiseptic [89–92]. Birch bark contains betulin, betulinol and a betuloside. The young leaves are rich in saponins and contain a diuretic flavonoid derivative (hyperoside), sesquiterpenes and tannins. The buds are also rich in volatile oil. Birch tar contains creosol and guaiacol[93–96].

Leaves and other parts of different birch species (Betula spp., Betulaceae), as well as products of birch such as the buds, bark, essential oil, juice, wood and tar, are used mainly for treating urinary tract disorders, severe infections and inflammations[97]. Birch leaves are especially popular as a remedy for progressive diuresis[98]. According to the European Medicines Agency, birch leaf is a traditional herbal medicinal product that increases the production of urine and helps flush the urinary tract by acting as an adjuvant for minor urinary complaints[99]. The chemical composition of flavonoids, as the main polyphenolic constituents of birch leaves, has been investigated quite extensively in recent years. *Betula Pendula Roth* or *Betula Pubescens* leaves contain the highest phenolic composition that may be important for the higher antioxidant concentration found in the extract. For example, Hänsel and Sticher (2007) identified the presence of the following flavonoids in birch leaves: quercetin-3-O-galactoside (=hyperoside), quercetin-3-O-glucuronide, myricetin-3-O-galactoside, quercetin-3-O-rhamnoside (=quercitrin), as well as other quercetin glycosides[100]. Quercetin and hyperoside were reported as the principal flavonoids in birch leaves by Evans (2000)[101].

According to the 7th European Pharmacopoeia, whole or fragmented dried *Betula Pendula* leaves, as well as the leaves of hybrids of both species, can be efficiently used for medical purposes. The leaves should contain not less than 1.5% of flavonoids, calculated as hyperoside, when used in a dried drug[102]. The birch leaves contain mainly polymeric proanthocyanidins; their total content (expressed as dry weight) is 39 mg/g in *Betula*

Pendula[103]. Carnat et al. (1996) analyzed the content of flavonoids in *Betula Pendula* and found total flavonoids 3.29 and 2.77%, hyperoside 0.80 and 0.77%, avicularin 0.57 and 0.26%, galactosyl-3 myricetol 0.37 and 0.18%, glucuronyl-3 quercetol 0.25 and 0.36%, and quercitrin 0.14 and 0.12%[104].

Field bindweed leaves (*Convolvulus Arvensis*)

Bindweed (*Convolvulus arvensis*) is a creeping weed widely distributed in the Middle East. It is often the dominant plant that grows under date palm trees in the eastern region of Saudi Arabia, where it is consumed by animals[105]. The root and the resin have cholagogue, diuretic, laxative and strongly purgative effects[106]. The tea from the flowers is a laxative and is also used in the treatment of fevers and wounds. A cold tea made from *Convolvulus arvensis* leaves is a laxative and is also taken as a herbal medicine to reduce excessive menstrual flow[107]. Although *Convolvulus arvensis* contains tropane alkaloids with an atropine-like action, it has rarely been associated with animal poisoning and may have a therapeutic value to animals due to its immunostimulation effect[108].

A high molecular-weight water extract can reduce the alkaloid content of *Convolvulus arvensis.* Meng et al. (2002) demonstrated that the alkaloid in *Convolvulus arvensis* is depleted during a high molecular-weight extraction. The plant also contains proteoglycan molecules (PGM), which can have anti-angiogenic, anti-tumor and immunostimulatory effects in human cells[109]. Hageb and Ghareib (2010) determined the phenolic content of *Convolvulus arvensis* methanol extract identifying the presence of pyrogallic acid, protocatechuic acid, ferulic acid and p-coumaric acid[111]. These compounds have been studied extensively and are responsible for many pharmacological effects on human health[112]. Sadeghi-aliabadi et al. (2008) determined the anti-cancer potential of *Convolvulus arvensis* (PGM) extract in its effect on human cancerous cells[113]. Meanwhile, Elzaawely and Tawata (2012) determined the total phenolic and flavonoid content of *Convolvulus arvensis* and evaluated its antioxidant capacity[114]. They showed a strong correlation between the phenolic compound and antioxidant activity, which may give

application for the phenolic fraction in the food industry. The study was also supported by Thakral et al. (2010)[115].

Common Bearberry leaves (*Arctostaphylos uva-ursi L. Sprengel*)

Common bearberry (*Arctostaphylos uva-ursi L. Sprengel*), known as kinnikinnick, belongs to the Ericaceae family, a ubiquitous trailing evergreen shrub often forming mats 50–100cm wide[116]. The plant grows preferentially on sandy and well-drained soil and is common in open woodlands, on rocky hills and on eroded slopes throughout the North American prairies, Asia and Europe[117]. The plant is an active ingredient in many commercial products and has an official classification as a phytomedicine in various parts of Europe[118]. Its commercial importance is based on its astringent properties and its beneficial effects on nephritis, kidney stones and other diseases of the urinary tract. The indigenous peoples of North America prepare a functional tea from bearberry for such a purpose[116]. The main compounds of bearberry leaf are arbutin (5–15%), methylarbutin (variable and up to 4%) and small quantities of the free aglycones. Its other constituents include ursolic acid, tannic acid, gallic acid, p-coumaric acid, syringic acid, galloylarbutin and up to 20% gallotannins, as well as some flavonoids, notably glycosides of quercetin, kaempferol and myricetin[119].

The bearberry plant is a tremendously underutilised renewable natural resource. Due to the presence of phenolic constituents, the bearberry plant is a warehouse of various bioactives. The antioxidant and antimicrobial effects of bearberry leaf extracts have been reported by several authors. The ethanolic extract from bearberry leaves exhibited high antioxidative activity in the inhibition of photo-induced chemiluminescence (PCL) of luminol reported by Pegg et al. (2007)[120]. Amarowicz et al. (1999, 2004) demonstrated the extract's very strong reducing power and its antioxidant properties in a β-carotene-linoleate model system. They also demonstrated its antiradical properties, which they investigated using the DPPH radical scavenging assay and an EPR spin-trapping technique[121,122]. The crude bearberry-leaf extract, as well as its low-molecular-weight phenolics and tannin fractions, inhibited TBARS formation in cooked pork systems after seven days of

refrigerated storage at a 200-ppm concentration[123]. The observed retardation in lipid oxidation/autoxidation by the bearberry leaf extract in cooked pork patties demonstrates the thermal stability of the bioactive constituents in the extract, which impart the antioxidant activity. In a study by Carpenter et al. (2007), the addition of the bearberry leaf extract decreased lipid oxidation (TBARS) in raw pork patties on days 9 and 12 of storage, relative to controls[60]. The inhibitory effects of bearberry leaf extracts against *Arcobacter butzleri, A. cryaerophilus* and *A. skirrowii* were reported by Cervenka et al. (2006)[124].

Amarowicz et al. (2013) demonstrated that the potential of low molecular weight phenolics (LMW fraction) from bearberry leaf extract inhibited the proliferation of human carcinoma cell lines. This means that the extract may have the potential to become an anti-cancer agent in drug development[118]. It has also been demonstrated that bearberry leaf extract is an anti-obesity agent due to its ability to interfere in fat hydrolysis, which reduces the utilization of ingested lipids and, therefore, inhibits lipases and decreases fat absorption [125]. The application of a bearberry leaf extract has been used in cosmetic products for skin lightening[126]. Up to this point, analysis of bioactive constituents in the bearberry leaf extract has been limited to thin-layer chromatography and high-performance thin-layer chromatography (HPTLC)[122]

2.3 Antioxidant effect in food models

Oil-in-water (O/W) emulsions

Oil-in-water (O/W) emulsions are lipid phases dispersed in an aqueous medium. They often occur in formulated foods. Such emulsions are stabilised by surface-active molecules adsorbed at the oil/water interface which are called surfactants. In the food industry, various surfactants are used to create emulsions. Surfactants are molecules with a hydrophobic (oil soluble) and an effective hydrophilic (water soluble) portion. They act as emulsifiers by significantly lowering the interfacial tension and decreasing the coalescence of dispersed droplets. From their manufacture to their end-use, including their dissolution in the digestive tract, food emulsions are subjected to a broad range of physical/chemical treatments. Under these conditions, and in the presence of oxygen, chemically reactive components may become oxidized. Among them, polyunsaturated fatty acids (PUFAs) are particularly prone to oxidation. Lipid oxidation has a deleterious effect on the technological, sensory, and nutritional qualities of food[127]. The reaction generates odorant compounds characterised by low detection thresholds and generally unpleasant aesthetic properties that damage the sensory quality of the products. Lipid oxidation also causes a loss of nutritional components and leads to the formation of free radicals and potentially toxic compounds[128].

In order to reduce and control lipid oxidation, antioxidants are added to foods. Synthetic antioxidants such as butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA) and ethylene diamine tetraacetic acid (EDTA) are used in the food industry to prevent the oxidation of food fat. These products are more economical than natural antioxidants but have negative connotations due to being chemical products. Plant materials rich in phenolic compounds have gained a lot of attention recently. The antioxidative effects of natural plant materials rich in phenolics, such as extracts of rosemary[129], berries[130], green tea[131], herbs[56,132,133], raisins[134] and olives[135], have been tested in a variety of O/W emulsions. Heinonen (2007) reported that anthocyanins isolated from black currants, raspberries and the juice of raspberries and blackberries had an inhibiting effect on lipid oxidation in O/W emulsions[130]. In another study, polar carotenoid (paprika, marigold,

bixin, norbixin) and hydrophobic (b-carotene, lycopene) carotenoids exerted a clear antioxidant effect during thermal accelerated autoxidation (60^0C) of o/w emulsions.

Meat

Meat is the muscle tissue of slaughtered animals and is composed of water, proteins, lipids, minerals and a small proportion of carbohydrates. It is susceptible to quality deterioration due to its rich nutritional composition[136]. Oxidation is one of the major causes of quality deterioration in meat due to the high concentrations of unsaturated lipids, heme pigments, metal catalysts and a range of oxidizing agents in muscle tissue. Oxidative deterioration in any type of meat manifests in the form of discolouration, development of an off flavour, the formation of toxic compounds, poor shelf life and nutrient and drip losses, respectively[137]. This quality deterioration is due to chemical and microbial changes. The most common form of chemical deterioration is the oxidation of meat lipids. Lipid oxidation is a complex process that depends on the chemical composition of meat, the presence of light and oxygen and the storage temperature[138]. It is also affected by some technological procedures to which meat is subjected during processing. It leads to the formation of several other compounds that have negative effects on the quality of meat and meat products and cause changes in sensory (colour, texture and flavor) and nutritional quality[139].

Lipid oxidation can be delayed by adding antioxidant sources to meat. The product quality and shelf-life can thereby be improved. Antioxidants can prevent lipid peroxidation through the following mechanisms: preventing chain inhibition by scavenging for initiating radicals, breaking chain reactions, decomposing peroxides, decreasing localised oxygen concentrations and binding chain initiating catalysts such as metal ions[140].

A huge number of compounds have been suggested as possessing antioxidant activity but only a few can be used in food products. The use of antioxidants in food products is controlled by countries' regulatory laws and international standards (Karre, Lopez, & Getty, 2013). The antioxidants can have synthetic or natural origins. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT),

tert-butylhydroquinone (TBHQ) and propyl gallate (PG) have been widely used in meat and poultry products[141]. However, the demand for natural antioxidants, especially of plant origin, has increased in recent years due to growing concerns among consumers about the potential toxicological effects of synthetic antioxidants[142]. A huge number of natural antioxidants have been studied in meat in recent years, as reviewed by Shah et al. (2014)[143]. These antioxidants have been extracted from different plant parts like leaves, roots, stems, fruits, seeds and bark. Some of these natural antioxidants are also available commercially and several studies have been carried out by different authors applying commercially available natural antioxidants of plant origin to meat[143]. Martín-Sánchez (2013) demonstrated that 10% (w/w) of fresh dates was enough to avoid lipid oxidation for 4 days of storage[144]. The effectiveness of a combination of oregano, sage and 5% (w/w) honey reduced the velocity of lipid oxidation in cooked chicken thighs and breasts after 48 and 96 h of refrigerated storage[145]. The use of a mixed vegetable powder of between 20%–30% w/w (spinach < yellow pea < onion < red pepper < green pea < tomato) improved the oxidative stability of turkey meat patties and decreased the formation of protein carbonyls, as reported by Dutthie et al. (2013). The addition of pomegranate rind, seed and juices caused a delay of the lipid oxidation process and improved the quality of cooked beef patties[146]. Meanwhile, when essential oil of rosemary marjoram and other oils were added to poultry meat at a concentration of 200 mg/kg, the lipid stability, anti-microbial effects and sensory traits of the meat were improved[147].

Active film packaging

Several strategies have been developed to reduce lipid oxidation and produce healthier and safer food products. These include adding direct natural antioxidant to foods and designing suitable packing technology. Vacuum or modified-atmosphere packaging combined with high-barrier packaging materials can limit the presence of oxygen. However, this approach does not always completely and effectively prevent the oxidation process due to the residual presence of oxygen at the time of packing or the permeation of oxygen through the package wall[148]. Moreover, some food products cannot be packed with less or no oxygen inside the packaging. The direct addition of antioxidant compounds to the

surface of foods may encounter a limitation once the active compounds are consumed in reaction, although some researchers believe that adding natural antioxidants directly can improve the nutritional quality of the food product[138,149]. Others have stated that the drawback of adding natural antioxidants directly is that it causes the protection and quality of the food to degrade at an increased rate[150,151]. Currently, antioxidant active packaging systems are being developed based on the incorporation of antioxidant agents in the package as a way to improve the stability of oxidation-sensitive food products.

Research studies have successfully demonstrated that the incorporation of natural antioxidant extracts from plants, spices and herbs in packaging, film and coatings can improve their active properties as antioxidants, antimicrobials and/or antibrownings agents[152–155]. The development of active packaging systems and edible films using natural additives has been studied in relation to different subjects such as the delay in lipid oxidation, redness of colour and antimicrobial properties[156,157]. Camo et al. (2008) studied lamb treated with antioxidant films that had been coated with oregano and showed that these films were significantly more efficient than those coated with rosemary. The fresh odour and colour of the food was also extended from 8 to 13 days compared to the control[150]. Ascorbic acid can be used in films as a browning inhibitor. The antibrowning effects of edible films and coatings containing ascorbic acid depend on the biopolymer type and the vitamin concentration added[158]. In addition, gelatine alone or constituted as a film exerts an antibrowning and antioxidant effect with natural coatings such as green tea, grape seed and basil leaf[155,159,160].

References

1. Darmanyan AP, Gregory DD, Guo Y, et al. Quenching of singlet oxygen by oxygen- and sulfur-centered radicals: evidence for energy transfer to peroxyl radicals in solution. *J Am Chem Soc*. 1998;120:396-403.

2. Heim KE, Tagliaferro AR, Bobilya DJ. Flavonoid antioxidants: chemistry, metabolism and structure–activity relationships. *J Nutr Biochem*. 2002;13:572-584.

3. Min DB, Boff JM. Chemistry and reaction of singlet oxygen in foods. *Comp Rev Food Sci F*. 2002;1:58-72.

4. Pokorny J. Are natural antioxidants better and safer that synthetic antioxidants? *Eur J Lipid Sci Technol*. 2007;109:629-642.

5. Kancheva VD. Phenolic antioxidants – radical-scavenging and chainbreaking activity: a comparative study. *Eur J Lipid Sci Technol*. 2009;111:1072-1089.

6. Carocho M, Ferreira ICFR. A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives. *Food Chem Toxicol*. 2013;51:15-25.

7. Pietta P. Flavonoids as antioxidants. *J Nat Prod*. 2000;63:1035-1042.

8. Ratnam DV, Ankola DD, Bhardwaj V, Sahana DK, Kumar NMVR. Role of antioxidants in prophylaxis and therapy: a pharmaceutical perspective. *J Control Release*. 2006;113:189-207.

9. Godman M, Bostick RM, Kucuk O, Jones DP. Clinical trials of antioxidants as cancer prevention agents: past, present and future. *Free Radic Biol Med*. 2011;51:1068-1084.

10. Rahman K. Studies on free radicals, antioxidants and co-factors. *Clin Inverv Aging*. 2007;2:219-236.

11. Ratnam DV, Ankola DD, Bhardwaj V, Sahana DK, Kumar NMVR. Role of antioxidants in prophylaxis and therapy: a pharmaceutical perspective. *J Control Release*. 2006;113:189-207.

12. Gamble PE, Burke JJ. Effect of water stress on the chloroplast antioxidant system. *Plant Physiol*. 1984;76:615-621.

13. Turunen M, Olsson J, Dallner G. Metabolism and function of coenzyme Q. *Biochim Biophys Acta*. 2004;1660:171-199.

14. Kandár R, Záková P, Muzáková V. Monitoring of antioxidant properties of uric acid in humans for a consideration measuring of levels of allantoin in plasma by liquid chromatography. *Clin Chim Acta*. 2006;365:249-256.

15. Steenvoorden DPT, Henegouwen GMJB. The use of endogenous antioxidants to improve photoprotection. *J Photochem Photobiol B*. 1997;41:1-10.

16. Barros AIRNA, Nunes FM, Gonçalves B, Bennett RN, Silva AP. Effect of cooking on total vitamin C contents and antioxidant activity of sweet chestnuts (Castanea sativa Mill.). *Food Chem*. 2011;128:165-172.

17. Burton GW, Traber MG. Vitamin E: antioxidant activity, biokinetics, and bioavailability. *Annu Rev Nutr*. 1990;10:357-382.

18. Brewer MS. Natural Antioxidants: Sources, Compounds, Mechanisms of Action, and Potential Applications. *Compr Rev Food Sci Food Saf*. 2011;10:221-247.

19. Tabassum A, Bristow RG, Venkateswaran V. Ingestion of selenium and other antioxidants during prostate cancer radiotherapy: a good thing? *Cancer Treat Rev*. 2010;36:230-234.

20. Procházková D, Boušová I, Wilhelmová N. Antioxidant and prooxidant properties of flavonoids. *Fitoterapia*. 2011;82:513-523.

21. Miller NJ, Sampson J, Candeias LP, Bramley PM, Rice-Evans C. Antioxidant activities of carotenes and xanthophylls. *FEBS Lett*. 1996;384(3):240-242.

22. Correia RTP, Borges KC, Medeiros MF, Genovese MI. Bioactive compounds and phenolic-linked functionality of powdered tropical fruit residues. *Food Sci Technol Int*. 2012;18:539-547.

23. Khokhar S, Magnusdottir SGM. Total phenol, catechin, and caffeine contents of teas commonly consumed in the United kingdom. *J Agric Food Chem*. 2002;50:565-570.

24. Krimmel B, Swoboda F, Solar S, Reznicek G. OH-radical induced degradation of hydroxybenzoic- and hydroxycinnamic acids and formation of aromatic products – a gamma radiolysis study. *Radiat Phys Chem*. 2010;79:1247-1254.

25. Terpinc P, Polak T, Šegatin N, Hanzlowsky A, Ulrih NP, Abramovic H. Antioxidant properties of 4-vinyl derivatives of hydroxycinnamic acids. *Food Chem*. 2011;128:62-68.

26. Gupta V, Sharma S. Plants as natural antioxidants. *Indian J Nat Prod Resour*. 2006;5:326-334. http://nopr.niscair.res.in/handle/123456789/7962.

27. Shahidi F, Zhong Y. Novel antioxidants in food quality preservation and health promotion. *Eur J Lipid Sci Technol*. 2010;112:930-940.

28. Moure Â, Cruz JM, Franco D, et al. Natural antioxidants from residual sources. *Food Chem*. 2001;72:145-171.

29. Kiselova Y, Ivanova D, Chervenkov T, Gerova D, Galunska B, Yankova T. Correlation between the in vitro antioxidant activity and polyphenol content of aqueous extracts from Bulgarian herbs. *Phytother Res*. 2006;20:961-965.

30. Geldof N, Engeseth NJ. Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of

in vitro lipoprotein oxidation in human serum samples. *J Agric Food Chem*. 2002;50:3050-3055.

31. Wong JW, Hashimoto K, Shibamoto T. Antioxidant Activities of Rosemary and Sage Extracts and Vitamin E in a Model Meat System. *J Agric Food Chem*. 1995;43:2707-2712.

32. Polovka M, Brezová V, Staško A. Antioxidant properties of tea investigated by EPR spectroscopy. *Biophys Chem*. 2003;106:39-56.

33. Azman NAM, Peiró S, Fajarí L, Julià L, Almajano MP. Radical scavenging of white tea and its flavonoid constituents by electron paramagnetic resonance (EPR) spectroscopy. *J Agric Food Chem*. 2014;62:5743-5748.

34. Boudjou S, Oomah BD, Zaidi F, Hosseinian F. Phenolics content and antioxidant and anti-inflammatory activities of legume fractions. *Food Chem*. 2013;138:1543-1550.

35. Khokhar S, Magnusdottir SGM. Total phenol, catechin, and caffeine contents of teas commonly consumed in the United kingdom. *J Agric Food Chem*. 2002;50:565-570.

36. Hilal Y, Engelhardt U. Characterisation of white tea – comparison to green and black tea. *J fur Verbraucherschutz und Leb*. 2007;2:414-421.

37. Qualities N. Antioxidant Action of Borage , Rosemary , Oregano , and Ascorbic Acid in Beef Patties. 2003;68:339-344.

38. Manchón N, Mateo-vivaracho L, Arrigo MD, et al. Distribution Patterns of Polyphenols and Alkaloids in Instant Coffee , Soft and Energy Drinks , and Tea. 2013;31:483-500.

39. Polyakov N. Carotenoids as antioxidants: spin trapping EPR andoptical study. *Free Radic Biol Med*. 2001;31:43-52.

40. Sánchez-Escalante A, Djenane D, Torrescano G, Beltrán JA, Roncalés P. The effects of ascorbic acid, taurine, carnosine and rosemary powder on colour and lipid stability of beef patties packaged in modified atmosphere. *Meat Sci*. 2001;58:421-429.

41. Azman NAM, Gordon MH, Skowyra M, Segovia F, Almajano MP. Use of lyophilised and powdered Gentiana lutea root in fresh beef patties stored under different atmospheres. *J Sci Food Agric*. 2014.

42. Cervantes-Paz B, Yahia EM, de Jesús Ornelas-Paz J, et al. Antioxidant activity and content of chlorophylls and carotenoids in raw and heat-processed Jalapeño peppers at intermediate stages of ripening. *Food Chem*. 2014;146:188-196.

43. Harrison EH. Mechanisms involved in the intestinal absorption of dietary vitamin A and provitamin A carotenoids. *Biochim Biophys Acta*. 2012;1821:70-77.

44. De Vogel J, Jonker-Termont DSML, van Lieshout EMM, Katan MB, van der Meer R. Green vegetables, red meat and colon cancer: chlorophyll prevents the cytotoxic and hyperproliferative effects of haem in rat colon. *Carcinogenesis*. 2005;26:387-393.

45. Velioglu YS, Mazza G, Gao L, Oomah BD. Antioxidant Activity and Total Phenolics in Selected Fruits, Vegetables, and Grain Products. *J Agric Food Chem*. 1998;46:4113-4117.

46. Wang SY, Lin HS. Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage. *J Agric Food Chem*. 2000;48:140-146.

47. Hassimotto NMA, Genovese MI, Lajolo FM. Antioxidant activity of dietary fruits, vegetables, and commercial frozen fruit pulps. *J Agric Food Chem*. 2005;53:2928-2935.

48. Kim S-J, Min SC, Shin H-J, et al. Evaluation of the antioxidant activities and nutritional properties of ten edible plant extracts and their application to fresh ground beef. *Meat Sci*. 2013;93:715-722.

49. Kim S-J, Cho AR, Han J. Antioxidant and antimicrobial activities of leafy green vegetable extracts and their applications to meat product preservation. *Food Control*. 2013;29:112-120.

50. Sharma SK, Singh L, Singh S. a Review on Medicinal Plants Having Antioxidant Potential. 2013;1:404-409.

51. Kusar A, Zupancic A, Sentjurc M, Baricevic D. Free radical scavenging activities of yellow gentian (Gentiana lutea L.) measured by electron spin resonance. *Hum Exp Toxicol*. 2006;25:599-604.

52. Mathew A, Taranalli AD, Torgal SS. Evaluation of Anti-inflammatory and Wound Healing Activity of Gentiana lutea Rhizome Extracts in Animals. 2008. Accessed March 17, 2014.

53. Muchuweti M, Kativu E, Mupure CH, Chidewe C, Ndhlala AR, Benhura MAN. . 2007. Phenolic composition and antioxidant properties of some spices. *Am J Food Technol*. 2007;2:414-420.

54. Lugasi A, Dworschak E, Hovari J. Characterization of scavenging activity of natural polyphenols by chemiluminescence technique. In: *Proceedings of the European Food Chemists VIII*. Vienna, Austria: Federation of the European Chemists' Society;1995:639-643.

55. Chen HY, Lin YC, C.L. H. Evaluation of antioxidant activity of aqueous extract of some selected nutraceutical herbs. *Food Chem*. 2007;104:1418-1424.

56. Gallego MG, Gordon MH, Segovia FJ, Skowyra M, Almajano MP. Antioxidant Properties of Three Aromatic Herbs (Rosemary, Thyme and Lavender) in Oil-in-Water Emulsions. *J Am Oil Chem Soc*. 2013;90:1559-1568.

57. Dorman HJD, Peltoketo a., Hiltunen R, Tikkanen MJ. Characterisation of the antioxidant properties of de-odourised aqueous extracts from selected Lamiaceae herbs. *Food Chem*. 2003;83:255-262.

58. Oliveira I, Sousa A, Ferreira ICFR, Bento A, Estevinho L, Pereira JA. Total phenols, antioxidant potential and antimicrobial activity of walnut (Juglans regia L.) green husks. *Food Chem Toxicol*. 2008;46:2326-2331.

59. Hegazy AE, Ibrahium MI. Antioxidant Activities of Orange Peel Extracts. 2012;18:684-688.

60. Carpenter R, O'Grady MN, O'Callaghan YC, O'Brien NM, Kerry JP. Evaluation of the antioxidant potential of grape seed and bearberry extracts in raw and cooked pork. *Meat Sci*. 2007;76:604-610.

61. J. P. *The Tea Companion: A Connoisseur's Guide*. 1st ed. Philadelphia, Pa: Running Press Book Publishers; 2004:160.

62. Almajano MP, Carbó R, Jiménez JAL, Gordon MH. Antioxidant and antimicrobial activities of tea infusions. *Food Chem*. 2008;108:55-63.

63. Obanda M, Owuor PO, Mang'oka R, Kavoi MM. Changes in thearubigin fractions and theaflavin levels due to variations in processing conditions and their influence on black tea liquor brightness and total colour. *Food Chem*. 2004;85:163-173.

64. Damiani E, Bacchetti T, Padella L, Tiano L, Carloni P. Antioxidant activity of different white teas: Comparison of hot and cold tea infusions. *J Food Compos Anal*. 2014;33:59-66.

65. Thring S, Hili P, Naughton D. Anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants. *BMC Complement Altern Med*. 2009;9:27.

66. Sohle J, Anja K, Holtzmann U, et al. White tea induces lipolytic activity and inhibits adipogenesis in human subcutaneous (pre)-adipocytes. *Nutr Metab*. 2009;6:20.

67. Moderno PM, Carvalho M, Silva BM. Recent patents on Camellia sinensis: source of health promoting compounds. *Recent Pat Food Nutr Agric*. 2009;1:182-192.

68. Cao H, Kelly M, Kari F, et al. Green tea increases anti-inflammatory tristetraprolin and decreases pro-inflammatory tumor necrosis factor mRNA levels in rats. *J Inflam*. 2007;14:1-12.

69. Almajano MP, Vila I, Gines S. Neuroprotective effects of white tea against oxidative stress-induced toxicity in striatal cells. *Neurotox Res*. 2011;20:372-378.

70. Yang C, Maliakal P, Meng X. Inhibition of carcinogenesis by tea. *Ann Rev Pharmacol Toxicol*. 2002;42:25-54.

71. López V, Calvo MI. White Tea (Camellia sinensis Kuntze) Exerts Neuroprotection against Hydrogen Peroxide-Induced Toxicity in PC12 Cells. *Plant Foods Hum Nutr*. 2011;66:22-26.

72. Koutelidakis A, Argiri K, Serafini M, et al. Green tea, white tea, and Pelargonium purpureum increase the antioxidant capacity of plasma and some organs in mice. *Nutrition*. 2009;25:453-458.

73. Sanatan-Rios G, Orner G, Amantana A, Provost C, Wu S, Dashwood R. Potent antimutagenic activity of white tea in comparison with green tea in salmonella assay. *Mutat Res Gene Toxicol Env Mutagen*. 2001;495:61-74.

74. Orner GA, Dashwood WM, Blum CA, Diaz GD, Li Q, Dashwood RH. Suppression of tumorigenesis in the Apcmin mouse: down-regulation of betacatenin signaling by a combination of tea plus sulindac. *Carcinogenesis*. 2004;24:263-267.

75. Gondoin A, Grussu D, Stewart D, McDougall GJ. White and green tea polyphenols inhibit pancreatic lipase in vitro. *Food Res Int*. 2010;43:1537-1544.

76. Karori SM, Wachira FN, Wanyoko JK, Ngure RM. Antioxidant capacity of different types of tea products. *Afr J Biotechnol*. 2007;6:2287-2296.

77. Unachukwu UJ, Ahmed S, Kavalier A, Lyles JT, Kennelly EJ. White and green teas (Camellia sinensis var. sinensis): Variation in phenolic, methylxanthine, and antioxidant profiles. *J Food Sci*. 2010;75:541-548.

78. Sultana T, Stecher G, Mayer R, et al. Quality assessment and quantitative analysis of flavonoids from tea samples of different origins by HPLC-DAD-ESI-MS. *J Agric Food Chem*. 2008;56:3444-3453.

79. Singh A. Phytochemicals of gentianaceae: a review of pharmacological properties. *Int J Pharm Sci Nanotechnol*. 2008;1:33-36.

80. Agency EM. *Assessment Report on Gentiana Lutea L., Radix, Doc. Ref.: EMA/HMPC/578322/2008, 2009.*:Doc. Ref.: EMA/HMPC/578322/2008, 2009.

81. Hänsel R, Sticher O. Pharmakognosie – Phytopharmazie. In: 8th ed. Springer Medizin Verlag, Heidelberg; 2007.

82. Aberham A, Schwaiger S, Stuppner H, Ganzera M. Quantitative analysis of iridoids, secoiridoids, xanthones and xanthone glycosides in Gentiana lutea L. roots by RP-HPLC and LC-MS. *J Pharm Biomed Anal*. 2007;45:437-442.

83. Oztürk N, Korkmaz S, Oztürk Y, Başer KHC. Effects of gentiopicroside, sweroside and swertiamarine, secoiridoids from gentian (Gentiana lutea ssp. symphyandra), on cultured chicken embryonic fibroblasts. *Planta Med*. 2006;72:289-294.

84. Recio MD, Giner RM, Manez S, Rios JL. Structural Considerations on the Iridoids as Antiinflammatory Agents. *Planta Med*. 1994;60:232-234.

85. Jiang D-J, Dai Z, Li Y-J. Pharmacological effects of xanthones as cardiovascular protective agents. *Cardiovasc Drug Rev*. 2004;22:91-102.

86. Schmieder A, Schwaiger S, Csordas A, et al. Isogentisin--a novel compound for the prevention of smoking-caused endothelial injury. *Atherosclerosis*. 2007;194:317-325.

87. Aberham A, Pieri V, Croom EM, Ellmerer E, Stuppner H. Analysis of iridoids, secoiridoids and xanthones in Centaurium erythraea, Frasera caroliniensis and Gentiana lutea using LC-MS and RP-HPLC. *J Pharm Biomed Anal*. 2011;54:517-525.

88. Heywood VH. *Flowering Plants of the World*. Oxford Uni. (Press OU, ed.). Oxford, UK; 1979:56.

89. Başer KHC. Studies on Betula essential oils. *Arkivoc*. 2007;vii:335-348.

90. Casetti F, Wölfle U, Gehring W, Schempp CM. Dermocosmetics for dry skin: A new role for botanical extracts. *Skin Pharmacol Physiol*. 2011;24:289-293.

91. Chambi F, Chirinos R, Pedreschi R, Betalleluz-Pallardel I, Debaste F, Campos D. Antioxidant potential of hydrolyzed polyphenolic extracts from tara (Caesalpinia spinosa) pods. *Ind Crops Prod*. 2013;47:168-175.

92. Germanò MP, Cacciola F, Donato P, et al. Betula pendula leaves: Polyphenolic characterization and potential innovative use in skin whitening products. *Fitoterapia*. 2012;83:877-882.

93. Wichtl M. *Herbal Drugs and Phytopharmaceuticals*. Medpharm S. (Bisset NG, ed.). Stuttgart, UK; 1994:106.

94. Guenther E. *The Essential Oils*. Vol. 2.,. Huntington, New York,: Robert E. Krieger, Publishing Co.; 1975:264.

95. Lawless J. *The Encyclopaedia of Essential Oils*. (Ltd EB, ed.). Longmead; 1992:59.

96. Kaneko N, Ishii H, Sato A, Kanisawa T, Watanabe S. 12th International Congress of Flavours, Fragrances and Essential Oils. In: Buchbauer G. E, ed. *Proceedings,Woidich, H.* Fachzeitschriftverlag-GmbH: Vienna,; 1992:53.

97. Emea. European Medicines Agency -. 2014;(May). http://www.ema.europa.eu/ema/.

98. Raal A, Boikova T, Püssa T. Content and Dynamics of Polyphenols in Betula spp . Leaves Naturally Growing in Estonia. 2015;1:41-48.

99. Raal A. *Birch. Medicinal Plants.* 28th ed. (N. J, Singh G and VK, eds.). Studium Press, Texas: Drug Plants II; 2010:121-142.

100. Hänsel R, Sticher O. *Pharmakognosie – Phytopharmazie*. 8th ed. (Aktuaslisierte überarbeitete und, Auflage, eds.). Springer, Heidelberg; 2007.

101. Evans WC. *Trease and Evans Pharmacognosy'*. 15th ed. Saunders, Edinburgh; 2000.

102. European Pharmacopoeia. 2010.

103. Vuorela S, Kreander K, Karonen M, et al. Preclinical evaluation of rapeseed, raspberry, and pine bark phenolics for health related effects. *J Agric Food Chem*. 2005;53:5922-5931.

104. Carnat A, Lacouture I, Fraisse D LJ-L. Standardisation de la feuille de bouleau. *Ann Pharm Françaises*. 1996;5:231-235.

105. Yeruham L, Shcosbery A. Poisionus Plant in Estern Mediterranean Basin. *Vet Hum Toxicol*. 2004;46:32-38.

106. Chopra RN, Nayar SL, Chopra IC. *Glossary of Indian Medicinal Plants (Including Supplement)*. New Delhi, India: CSIR; 1986.

107. Foster S, Duke JA. *A Field Guide of Medicinal Plants*. New York, USA: Houghton Mifflin Co.; 1990.

108. Al-Bowait ME, Albokhadaim IF, Homeida AM. Immunostimulant Effect of Binweed (Convolvulus Arvensis) Extract in Rabbit. *Res J Pharmacol*. 2010;4:51-54.

109. Meng XL, Riordan NH, Casciari JJ, Zhu Y, Zhong J, González MJ, Miranda-Massari JR, Riordan HD. Effects of a high molecular mass *Convolvulus arvensis* extract on tumor growth and angiogenesis. *P P R Health Sci J*. 2002;21:323-8.

110. Riordan NH, Meng X, Riordan HD. Anti-Angiogenic, anti-tumor and Immunostimulatory effect of a non-toxic plant extract (PGM). *Comprehensive Cancer Care.* 2000.

111. Hegab MM and Ghareib HR. Methanol extract potential of field bindweed (Convolvulus Arvensis L.) for wheat growth enhancement. Int Journal of Botany. 2010;6:334-342.

112. Kiliç I, Yeşiloğlu Y. Spectroscopic studies on the antioxidant activity of p-coumaric acid. *Spectrochim Acta A Mol Biomol Spectrosc*. 2013;115:719-724.

113. Sadeghi-aliabadim H, Ghasemi N and Kohi M. Cytotoxic effect of Convolvulus arvensis extracts on human cancerous cell line. Res Pharm Sci. 2008;3:31-34

114. Elzaawely AA, Tawata S. Antioxidant Activity of Phenolic Rich Fraction Obtained from Convolvulus arvensis L. leaves Grown in Egypt. *Asian J Crop Sci*. 2012;4:32-40.

115. Thakral J, Borar S, Kalia AN. Antioxidant Potential Fractionation from Methanol Extract of Aerial Parts of Convolvulus arvensis Linn . (Convolvulaceae). 2010;2:219-223.

116. Willard T. *Bearberry*. In Edible . Calgary , AB: Wild Rose College of Natural Healing,Ltd; 1992:164-165.

117. Simonot DL. *Bio-Manufacturing in Saskatche Wan. In Assessm Ent of the Manufacturing Potential of Select Saskat Chew an Plants*. Sasatoon, SK: Saskatche wan Nutraceutical Network; 2000:23-26.

118. Amarowicz R, Pegg RB. Inhibition of proliferation of human carcinoma cell lines by phenolic compounds from a bearberry-leaf crude extract and its fractions. *J Funct Foods*. 2013;5:660-667.

119. Barl B, Loewen D, Svendsen E. *Arctostaphylos Uv a-Ursi L. Spreng*. In Saskatc. Saskatoon, SK: University of Saskatchewan; :18-23.

120. Pegg RB, R. A, Naczk M, Shahidi F. *PHOTOCHEM_ for Determination of Antioxidant Capacity of Plant Extract. In Antioxidant Measurement and Applications*. Washington, DC: American Chemical Society; 2007:140-158.

121. Amarowicz R, Pegg RB, Rahimi-Moghaddam P, Barl B, Weil JA. Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. *Food Chem*. 2004;84:551-562.

122. Amarowicz R, Barl B, Pegg RB. Potential, natural antioxidants from saskatchewan indigenous plants. 1999;6:306.

123. Pegg RB, Amarowicz R, Naczk M. Antioxidant activity of polyphenolics from abearberry-leaf (Ar ctostaphylos uv a-ursi L. Sprengel)extract in meat syste ms. Phenolic compounds in foods and natural health products. In: *ACS Symp Osium Series 909*. Washington , DC: In F.Shahidi &C.-T . Ho (Eds.); 2005:67-82.

124. Cervenka L, Peskova I, Foltynova E, Pejchalova M, Brozkova I, Vytrasova J. Inhibitory effects of some spice and herb extracts against Arcobacter butzleri, A. cryaerophilus, and A. skirrowii. *Curr Microbiol*. 2006;53:435-439.

125. Slanc P, Doljak B, Kreft S, Lunder M, S DJ, Borut Í. Screening of Selected Food and Medicinal Plant Extracts for Pancreatic Lipase Inhibition. 2009;877:874-877.

126. Marks A. *Herbal Extracts in Cosmetics.* 8th ed. Agro Food Industry Hi- Tech; 1997:28-31.

127. Frankel EN. *Lipid Oxidation*. Bridgwater, England: The Oily Press LTD; 2005:486.

128. Petersen DR, Doorn JA. Reactions of 4-hydroxynonenal with proteins and cellular targets. *Free Radic Biol Med*. 2004;37:937-945.

129. Ahn J-H, Kim Y-P, Kim H-S. Effect of natural antioxidants on the lipid oxidation of microencapsulated seed oil. *Food Control*. 2012;23:528-534.

130. Heinonen M. Antioxidant activity and antimicrobial effect of berry phenolics-a finnish perspective. *Mol Nutr Food Res*. 2007;51:684-691.

131. Almajano MP, Gordon MH. Synergistic effect of BSA on antioxidant activities in model food emulsions. *Am Oil Chem Soc*. 2004;81:275-280.

132. Skowyra M, Falguera V, Gallego G, Peiró S, Almajano MP. Antioxidant properties of aqueous and ethanolic extracts of tara (Caesalpinia spinosa) pods in vitro and in model food emulsions. *J Sci Food Agric*. 2013; 94:911-8.

133. Azman N, Segovia F, Martínez-Farré X, Gil E, Almajano M. Screening of Antioxidant Activity of Gentian Lutea Root and Its Application in Oil-in-Water Emulsions. *Antioxidants*. 2014;3(2):455-471.

134. Williamson G, Carughi A. Polyphenol content and health benefits of raisins. *Nutr Res*. 2010;30:511-519.

135. Mattia CDD, Sacchetti G, Mastrocola D, Pittia P. Effect of phenolic antioxidants on the dispersion state and chemical stability of olive oil O/W emulsions. *Food Res Int*. 2009;42:1163-1170.

136. Devatkal SK, Naveena BM. Effect of salt, kinnow and pomegranate fruit by-product powders on color and oxidative stability of raw ground goat meat during refrigerated storage. *Meat Sci*. 2010;85:306-311.

137. Contini C, Alvarez R, O'Sullivana M, Dowling DP, Gargan SO, Monahan FJ. Effect of an active packaging with citrus extract on lipid oxidation and sensory quality of cooked turkey meat. *Meat Sci*. 2014;96:1171-1176.

138. Doménech-Asensi G, García-Alonso FJ, Martínez E, et al. Effect of the addition of tomato paste on the nutritional and sensory properties of mortadella. *Meat Sci*. 2013;93:213-219.

139. Karakaya M, Bayrak E, Ulusoy K. Use of natural antioxidants in meat and meat products. *J Food Sci Eng*. 2011;1:1-10.

140. Dorman HJD, Peltoketo A., Hiltunen R, Tikkanen MJ. Characterisation of the antioxidant properties of de-odourised aqueous extracts from selected Lamiaceae herbs. *Food Chem*. 2003;83:255-262.

141. Formanek Z, Lynch A, Galvin K, Farkas J, Kerry JP. Combined effects of irradiation and the use of natural antioxidants on the shelf life stability of over wrapped minced beef. *Meat Sci*. 2003;63:433-440.

142. Jayathilakan K, Sharma GK, Radhakrishna K, Bawa AS. Antioxidant potential of synthetic and natural antioxidants and its effect on warmed-over-flavour in different species of meat. *Food Chem*. 2007;105:908-916.

143. Shah MA, Bosco SJD, Mir SA. Plant extracts as natural antioxidants in meat and meat products. *Meat Sci*. 2014;98:21-33.

144. Martín-Sánchez AM, Ciro-Gómez G, Sayas E, Vilella-Esplá J, Ben-Abda J, Pérez-Álvarez JÁ. Date palm by-products as a new ingredient for the meat industry: application to pork liver pâté. *Meat Sci*. 2013;93(4):880-887.

145. Sampaio GR, Saldanha T, Soares R a M, Torres E a FS. Effect of natural antioxidant combinations on lipid oxidation in cooked chicken meat during refrigerated storage. *Food Chem*. 2012;135:1383-1390.

146. Ibrahim HM, Moawad RK, Emam WH. Antioxidant Effect of Pomegranate Rind , Seed Extracts and Pomegranate Juice on Lipid Oxidation and Some Quality Properties of Cooked Beef patties. 2012;8:4023-4032.

147. Mohamed HMH, Mansour H a. Incorporating essential oils of marjoram and rosemary in the formulation of beef patties manufactured with mechanically deboned poultry meat to improve the lipid stability and sensory attributes. *LWT - Food Sci Technol*. 2012;45:79-87.

148. López-de-Dicastillo C, Alonso JM, Catalá R, Gavara R, Hernández-Muñoz P. Improving the antioxidant protection of packaged food by incorporating natural flavonoids into ethylene-vinyl alcohol copolymer (EVOH) films. *J Agric Food Chem*. 2010;58:10958-10964.

149. Hayes JE, Stepanyan V, Allen P, O'Grady MN, Kerry JP. Effect of lutein, sesamol, ellagic acid and olive leaf extract on the quality and shelf-life stability of packaged raw minced beef patties. *Meat Sci*. 2010;84:613-620.

150. Camo J, Beltrán JA, Roncalés P. Extension of the display life of lamb with an antioxidant active packaging. *Meat Sci*. 2008;80:1086-1091.

151. De Abreu D a. P, Rodriguez KV, Cruz JM. Extraction, purification and characterization of an antioxidant extract from barley husks and development of an antioxidant active film for food package. *Innov Food Sci Emerg Technol*. 2012;13:134-141.

152. Bentayeb K, Rubio C, Batlle R, Nerín C. Direct determination of carnosic acid in a new active packaging based on natural extract of rosemary. *Anal Bioanal Chem*. 2007;389:1989-1996.

153. Contini C, Katsikogianni MG, O'Neill FT, O'Sullivan M, Dowling DP, Monahan FJ. PET trays coated with Citrus extract exhibit antioxidant activity with cooked turkey meat. *LWT - Food Sci Technol*. 2012;47:471-477.

154. Wang S, Marcone M, Barbut S, Lim LT. The Impact of Anthocyanin-Rich Red Raspberry Extract (ARRE) on the Properties of Edible Soy Protein Isolate (SPI) Films. *J Food Sci*. 2012;77:497-505.

155. Siripatrawan U, Harte BR. Physical properties and antioxidant activity of an active film from chitosan incorporated with green tea extract. *Food Hydrocoll*. 2010;24:770-775.

156. Iturriaga L, Olabarrieta I, de Marañón IM. Antimicrobial assays of natural extracts and their inhibitory effect against Listeria innocua and fish spoilage bacteria, after incorporation into biopolymer edible films. *Int J Food Microbiol*. 2012;158:58-64.

157. Jongjareonrak A, Benjakul S, Visessanguan W, Tanaka M. Antioxidative activity and properties of fish skin gelatin films incorporated with BHT and ??-tocopherol. *Food Hydrocoll*. 2008;22:449-458.

158. Ouattara B, Girouxa M, Yefsah R, et al. Microbiological and biochemical characteristics of ground beef as affected by gamma irradiation, food additives and edible coating film. *Radiat Phys Chem*. 2002;63:299-304.

159. Li JH, Miao J, Wu JL, Chen SF, Zhang QQ. Preparation and characterization of active gelatin-based films incorporated with natural antioxidants. *Food Hydrocoll*. 2014;37:166-173.

160. Tongnuanchan P, Benjakul S, Prodpran T. Structural, morphological and thermal behaviour characterisations of fish gelatin film incorporated with basil and citronella essential oils as affected by surfactants. *Food Hydrocoll*. 2014;41:33-43.

CHAPTER 3

CHAPTER 3: RESULTS AND DISCUSSION

3.1. Radical Scavenging of White Tea and of its Flavonoid Constituents by EPR Spectroscopy

Abstract

White tea (WT), presents high levels of catechins, known to reduce oxidative stress. WT is the least processed tea, unfermented, prepared only from very young tea. The aim of this article is the use of the spin trap method and the electron paramagnetic resonance (EPR) spectroscopy as the analytical tool to measure, for the first time, the radical scavenging activity of WT and its major catechin components, epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG), against the methoxy radical using Ferulic acid as antioxidant pattern. The antioxidant activity has been measured by the decrease of the intensity of the spectral bands of the adduct DMPO-OCH_3 in the EPR with the amount of antioxidant in the reactive mixture. The tea leaves and buds were extracted from waterless Methanol. It has been proved that tea compounds with more antiradical activity against methoxy radical are those with gallate group, EGCG and ECG.

Keywords:

White tea, EPR, spin trap, antioxidant activity, radical scavenging

3.1.1 Introduction

Tea (*Camellia sinensis (L.)*) is one of the world's most widely consumed beverages, and its medicinal properties have been widely explored. It can be classified in three types: Unfermented (green and white teas), partially fermented (oolong tea) and completely fermented (black tea).[1] White tea (WT) is exclusively prepared from young tea leaves or buds, harvested before being fully opened. It is the least processed tea, not having suffered from any fermentation process; leaves are only air-dried.[2–4] The strong antioxidant capacity of WT is linked to its high catechins content. The major catechins of tea are: epigallocatechin (EGC), catechin (C), epigallocatechin gallate (EGCG), epicatechin (EC) and epicatechin gallate (ECG).[2,5] Scavenging capacity of WT can be measured by different antioxidant tests such as ORAC, TEAC, FRAP or DPPH, among others.[6] Nowadays, WT is also used as a promising antioxidant medium additive, even better than green tea.[7] It is also suggested that the mechanism of the protective actions of WT in oxidative stress in vitro, is related to its antioxidant potential and the maintenance of the normal redox status of the cell, when it is attacked by radicals or H_2O_2.[8]

In this context, the radical scavenging measurements by traditional methods have their limitations. An essential antioxidant test extensively used in the literature, deals with the scavenging activity of antioxidants against the hydroxyl radical ($HO^\bullet$) or the superoxide ($O_2^\bullet$), which are the main harmful reactive oxygen species (ROS). The activity of the antioxidant is measured in a competitive reaction with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), an excellent $HO^\bullet$ and $O_2^{\bullet-}$ spin trap nitrone. Spin trapping is a technique in which a transient radical, very unstable under normal conditions, react with a spin traps to form a much more stable radical adduct detected by electron paramagnetic resonance (EPR) spectroscopy. Nitrones have been the molecules of choice as spin trap species due to their specificity and ability to quantify transient ROS. It is well known that the spin trapping technique can provide direct evidence of the presence of transient radicals in any reaction system and therefore, it is very useful for discriminating trapped radical species, and various short-lived radical intermediates have been identified by this technique.[9,10] Thus, the EPR spectroscopy has been an indispensable tool for the detection of many harmful ROS ($O_2^{\bullet-}$, $HO^\bullet$, $HO_2^\bullet$, $RO_2^\bullet$, $RO^\bullet$), via spin trapping.[10–13] Thereby, the antioxidant activity

of a single polyphenol or a natural extract (mixture of polyphenols), is measured by the decrease of the intensity of the spectra of the radical adduct in the presence of the antioxidant relative to the decrease in the presence of an antioxidant pattern.[14–22]

Some studies have been reported about the radical scavenging activity of green and black teas against 2,2-diphenyl-1-picrylhydrazyl (DPPH) by using the EPR technique.[10,23–27] However, there are no publications about the use of EPR in the analysis of the antioxidant capacity of WT with a transient oxygen radical, much more unstable than DPPH.

Following our research work related to the antioxidant and antimicrobial properties of WT,[28–30] the aim of this article is to measure the radical scavenging activity of WT and its major constituents *i.e.* epicatechin (EC), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG), against methoxy radical, generated in the Fenton reaction in MeOH as solvent, using Ferulic acid as antioxidant pattern. The alcoxy radicals are considered among ROS species in biological processes, as also critical mediators in several serious human diseases. The classical Fenton reaction in aqueous media is used in most cases to generate the harmful hydroxyl radical $HO^{\bullet}$.. However, when the reduction of H_2O_2 with $FeSO_4$ is conducted in MeOH as solvent, the transient radical generated is the methoxy radical $CH_3O^{\bullet}$ as discussed below.

3.1.2 Materials and Methods

Materials

Commercial available WT was obtained from local market. Ferulic acid, epicatechin-3-gallate (ECG), epigallocatechin (EGC), epigallocatechin-3-gallate (EGCG) and epigallocatechin (EC) were bought at Sigma Aldrich. Iron (II) sulfate ($FeSO_4$), 5,5-Dimethyl-1-pyrroline-N-oxide (DMPO), hydrogen peroxide (H_2O_2) and methanol (MeOH) were analytical grade and supplied by Panreac (Barcelona, Spain).

Extraction and quantification of WT

The extraction was made in two different ways.

a. Dried WT (10 g) were mixed with MeOH (100 mL) and extracted for 24 h at 4 ± 1 ºC, stirred (10^3 rpm) and light protected.

b. Dried WT (10 g) were mixed with hot Water (100 mL) and extracted for 4 min at 90 ± 1 ºC, stirred (10^3 rpm) and light protected.

In both cases, the resulting infusions were filtered with qualitative filter papers (Watmann n.1) in a vacuum system. In order to determine the soluble concentration of WT, the solvent was removed at low pressure (BUCHI RE111, Switzerland) and further treated in a freeze dryer (Unicryo MC2L +/-60°C, Germany). Finally, WT lyophilized pulps were weighted and the concentration recovered from extract (g/l) was determined. The lyophilized extracts were kept in a desiccator, protected from light, until analysis. An adequate dilution of each extract was prepared to analyze in HPLC method to know the concentration of different flavonoids. The results of the main catechins for the water extraction are EC 51.3 ± 3.7; EGC 157 ± 9.5; EGCG 1631 ± 97; ECG 280 ± 23.2 mg/100 g tea dry leaves. In methanol, from 100 g of dry WT an average of 7.5 ± 0.2 g of soluble extract were collected. The major phenolic compounds found in methanolic WT extracts by HPLC analysis are listed in Table 1.

Table 1: Concentration of Polyphenols Found in MeOH WT Extracts by HPLC Analysis.

Compound	Retention Time (min)	Concentration (mg/100 g dry tea leaves)
EGC	1.913	260.9
EC	2.91	94.9
EGCG	4.715	2113.8
ECG	7.757	382.2

Determination of methoxy radical scavenging activity by EPR

The solutions of the different components were prepared in deoxygenated MeOH. A spin-trapping reaction mixture consisted of 100 μL of DMPO (35 mM), 50 μL of H_2O_2 (10 mM), 50 μL of pure polyphenols EC, ECG, EGC, EGCG at different concentrations or WT extract (0-8.13 g/L) or 50 μL of Ferulic acid used as reference (0-20 g/L) or 50 μL of pure MeOH used as a control, and finally 50 μL of $FeSO_4$ (2 mM), added in that order. The final solutions (250 μL) were passed to a narrow (inside diameter, 2 mm) quartz tube, introduced into the cavity of the EPR spectrometer and the spectrum was recorded 10 min after the addition of the $FeSO_4$ solution, when the radical adduct signal is greatest.

X-band EPR spectra were recorded with a Bruker EMX-Plus 10/12 spectrometer under the following conditions: microwave frequency, 9.8762 GHz; microwave power, 30.27 mW; center field, 3522.7 G; sweep width, 100 G; receiver gain, 5.02 x 10^4; modulation frequency, 100 kHz; modulation amplitude, 1.86 G; time constant, 40.96 msec; conversion time, 203.0 msec.

Each measurement was carried out in triplicate. The first derivative of the absorption signal was twice integrated, resulting values directly proportional to the concentration of the remaining radical adducts, when the competitive reactions of the methoxy radical with DMPO and the antioxidant are completed. Then, these values are compared with those obtained with Ferulic acid.

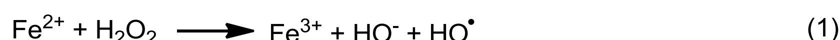
3.1.3 Results and Discussion

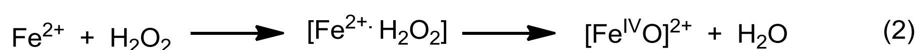
Antioxidant capacity assay

In the present study, the free radical scavenging activity of WT extracts was evaluated against methoxy ($CH_3O^\bullet$) radical by a competitive method in the presence of DMPO as spin trap, using EPR spectroscopy. $CH_3O^\bullet$ radical was generated according to the Fenton procedure in MeOH instead of H_2O as solvent. Methoxy radical, with a relatively

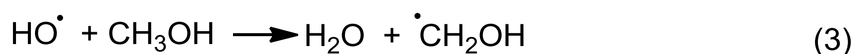
short half-life, has been identified by EPR because of its ability to form a stable nitroxide adduct with DMPO, DMPO-OCH_3 (hyperfine splitting constants, a_N = 13.9 G and a_H = 8.3 G).[31]

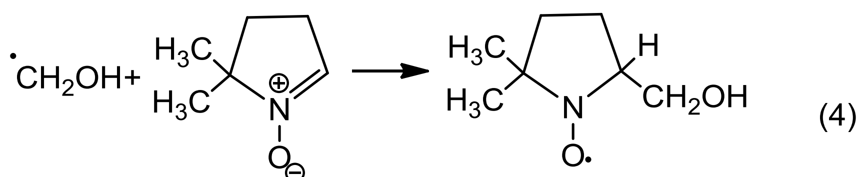
Since iron has a variable valence, its oxidation by H_2O_2 may occur via one or two electron transfer reactions. Therefore, two different mechanisms have been extensively discussed in the literature about the Fenton reaction.[32–39] A radical mechanism with Fe(II) salt as one-electron donor and the generation of hydroxyl ($HO^{\bullet}$) radical (reaction 1),[40] and a non-radical mechanism which involves a direct interaction between the Fe(II) and H_2O_2. This interaction finally produces an iron-oxide species of Fe(IV) (reaction 2).[41] In each case, the predominance of one mechanism over the other may depend on different variables, mainly the nature of the metal ligand, the solvent and the pH of the medium.





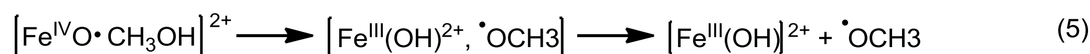
As said above, our experiments have been carried out in MeOH due to the low solubility of Ferulic acid in water. Assuming a radical mechanism in the Fenton reaction in MeOH, the generated hydroxyl radical would abstract a hydrogen atom mainly from the α position of MeOH to yield a hydroxymethyl radical (reaction 3)[42] which, in turn, would react with DMPO to yield the radical adduct DMPO-CH_2OH[43] (reaction 4) (hyperfine splitting constants, a_N = 14.7 G and a_H = 20.7 G).[44]

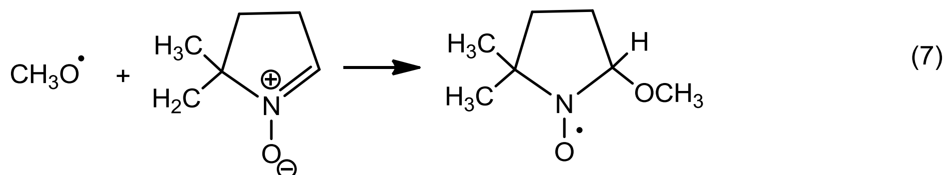




However, in the reduction process of H_2O_2 with Fe(II) in MeOH as solvent in the presence of DMPO as spin trap, the unique bands of significant intensity shown in the EPR spectrum correspond to those of the methoxy adduct, DMPO-OCH_3, and no relevant bands

to the DMPO adducts with hydroxyl and α-hydroxymethyl radicals are detected. Therefore, we assume a non-radical process as the dominant mechanism in the Fenton reaction in these conditions (reaction 2), and we suggest that the oxidant action of Fe(IV) oxide in MeOH leads to the generation of methoxy radical in a two consecutive one-electron paths (reactions 5 and 6) yielding in the presence of the spin trap, the radical adduct DMPO-OCH_3 (reaction 7). At this point, it should be emphasized that the involvement of an ionic mechanism in the formation of the radical adduct DMPO-OCH_3 by nucleophilic addition of MeOH, is discarded because the intensity of the EPR spectrum decreases by the addition of methoxy radical scavengers.





Pure polyphenols or WT extracts added to methanolic solutions of hydrogen peroxide (H_2O_2) and small amounts of $FeSO_4$ in the presence of an excess of DMPO, may compete with the spin trap in the scavenging of methoxy radicals. This effect decreases the amount of radical adducts and, accordingly, decreases the intensity of the EPR signal. The double integration value of the signal of the spectrum for each experiment in the presence of an amount of antiradical relative to the value in the presence of the same amount of Ferulic acid gives a measure of the antiradical activity of the test sample.

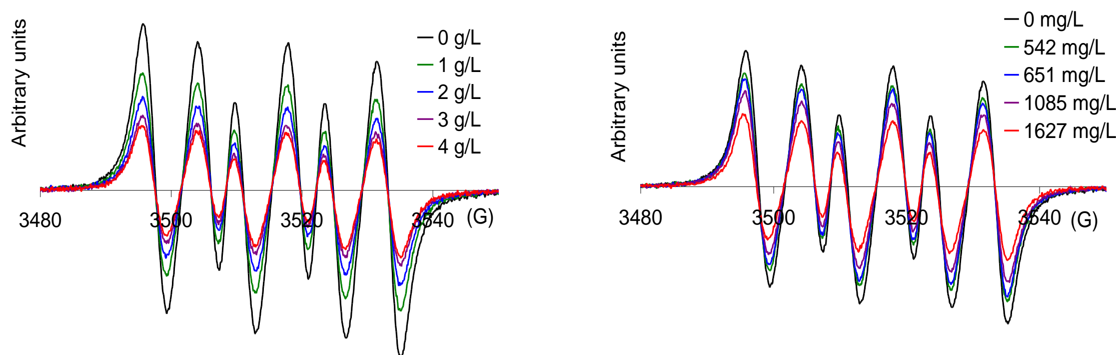


Figure 1. Electron paramagnetic resonance (EPR) spectra of the radical adduct DMPO-OCH_3 generated from a solution of H_2O_2 [2·mM] and $FeSO_4$ [0.04 mM] with DMPO [14 mM] as spin trap in MeOH as solvent. Left: decrease of the EPR signal with the increase of the concentration of Ferulic acid as the reference sample. Right: decrease of the EPR signal by increasing the concentration of the white tea extracts. EPR measurement conditions were as described in Materials and Methods section.

Figure 1 shows the EPR spectra of DMPO adduct of methoxy radical at different concentrations of Ferulic acid. The spectra were recorded 10 min after the addition of $FeSO_4$ to the reaction mixture, when the radical adduct signal was the greatest. The signal intensity of the spectrum decreased with the increase of the amount of Ferulic acid. Table 2 shows the values of the double integration at five different concentrations of Ferulic acid.

Table 2. Methoxy Radical Scavenging Activity of Ferulic Acid by the Spin Trap Technique with DMPO in EPR[a]

Ferulic acid (g/L)[b]	Ferulic acid (mM)[b]	DI EPR signal[c]
0	0	28.26
1	5.15	21.23
2	10.30	16.35
3	15.45	13.55
4	20.60	12.13

[a]Final concentration values of DMPO (14 mM), H_2O_2 (2 mM) and $Fe(SO_4)$ (0.4 mM) in 250 μL. [b]Final concentration values of Ferulic acid in 250 μL.[c]Double integral of the EPR signal.

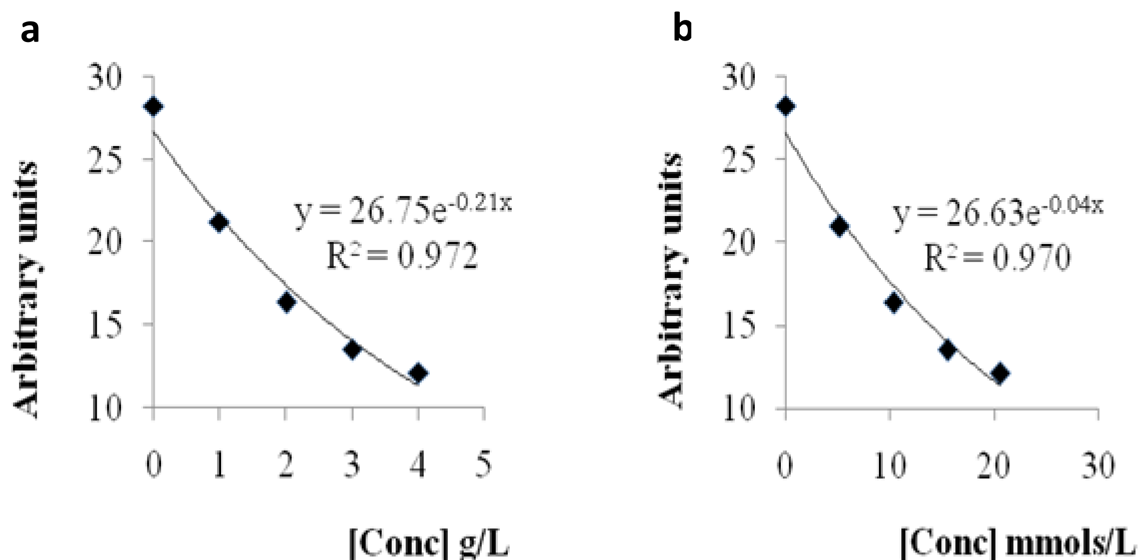


Figure 2. Decrease of the intensity of the spectrum of the spin adduct of methoxy radical with DMPO by increasing concentration of Ferulic acid. The best fitting function is an exponential curve with the equation given inside the graphic, a) in g/L and b) in mmols/L, and in the text.

The best fitting with these data was an exponential function (Figure 2) that, if concentration values of Ferulic acid are in g/L, corresponds to equation (1):

$$y = 26.75e^{-0.21x};\ R^2 = 0.972 \qquad (1)$$

The evolution of the signal intensity of the DMPO adduct in the presence of different amounts of WT extracts is shown in Figure 1. The radical scavenging activity values of WT extracts against methoxy radical are presented in Table 3 as the equivalents of Ferulic acid in grams per liter per gram of WT. The scavenging activity of the WT extract is evaluated from four different concentrations of the extract. The last column in Table 3 shows that the activity of the extract is slightly higher than the activity of Ferulic acid in grams per liter.

Table 3.Methoxy Radical Scavenging Activity of WT Extracts by the Spin Trap Technique with DMPO in the EPR, Measured as Ferulic Acid (FA) Equivalents per Gram of the Active Sample[a]

WT conc.(mg/L)[b]	DI EPR signal[c]	FA equivalents (mg/L)[d]	FA equi: WT[e]
0	28.26		
542	23.66	706.67	1.30
651	22.64	876.67	1.35
1085	20.70	1200.00	1.11
1627	14.94	2160.00	1.33

[a] Final concentration values of DMPO (14 mM), H_2O_2 (2 mM) and $Fe(SO_4)$ (0.4 mM) in 250 µL. [b]White tea extracts final concentration in 250 µL. [c] Double integral of the EPR signal. [d] Ferulic acid equivalents in mg/L. [e] Ferulic acid equivalents in g/L per gram of white tea extract.

The radical scavenging activity of pure polyphenols towards methoxy radical was examined. Solutions of H_2O_2 (2 mM) and $Fe(SO_4)$ (0.4 mM) in the presence of DMPO (14 mM), and varying amounts of polyphenol were introduced into the EPR cavity and the double integration of the spectrum obtained in 10 min after the addition of the Fe(II) salt into the reaction mixture was collected to calculate results. The overall chemical process can be defined as a system of two competing reactions between DMPO and the pure antioxidant over the methoxy radical. The action of the spin trap is to capture the methoxy radical to yield the radical adduct and, on the other hand, the scavenging action of the antioxidant on the methoxy radical reduces the amount of the radical adduct. In each experiment, the amount of spin trap was constant and the amount of antioxidant was varied. Then, the amount of radical adduct decreases exponentially as the amount of polyphenol increases, and the antioxidant efficiency is measured by the exponential coefficient α of the equation (2)

$y = y_0 \cdot e^{-\alpha x}$ (2)

where y is the value of the double integration of the spectrum of DMPO-OCH_3 at a given polyphenol concentration and y_0 represents the value of the double integration in the absence of polyphenol. The variable x represents the polyphenol concentration.

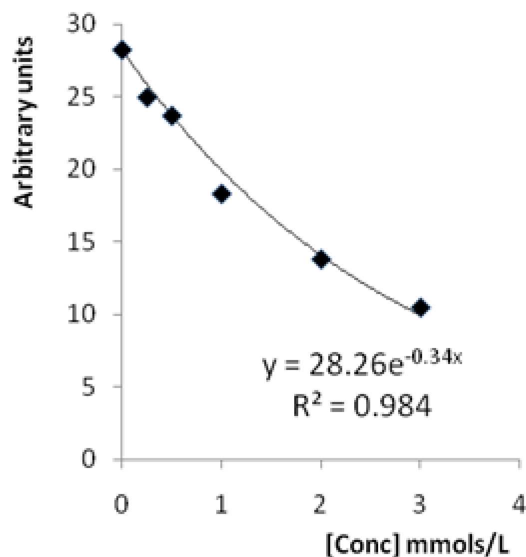


Figure 3. Decrease of the intensity of the spectrum of the spin adduct of methoxy radical with DMPO by increasing concentration of **EC**. The best fitting function is an exponential curve with the equation given inside the graphic and in the text.

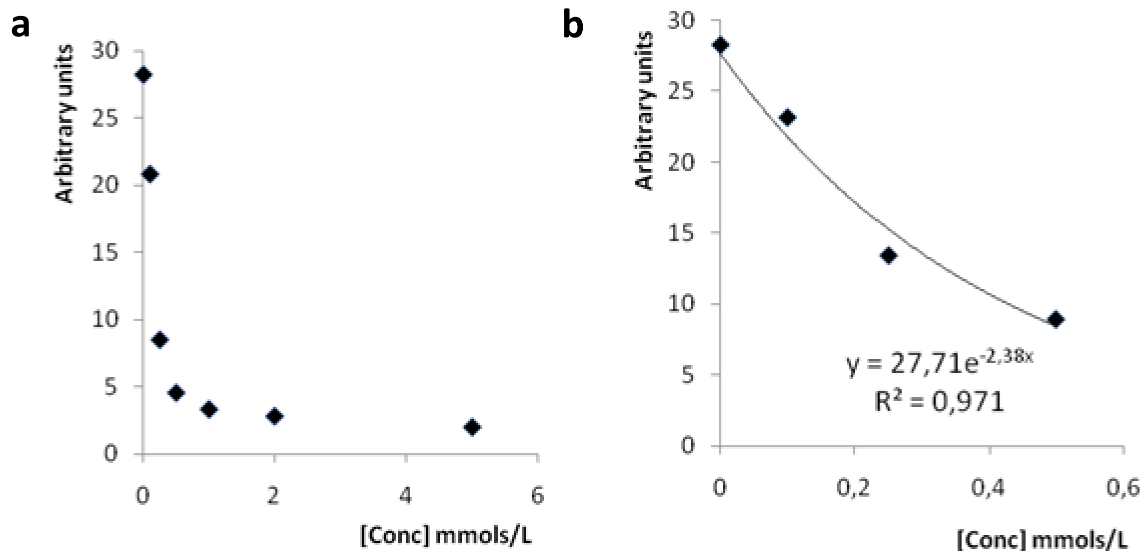


Figure 4. (a) Decrease of the intensity of the spectrum of the spin adduct of methoxy radical with DMPO by increasing concentration of **ECG**. (b) Detail of the first values of the graph; the best fitting function is an exponential curve with the equation given inside the graphic and in the text.

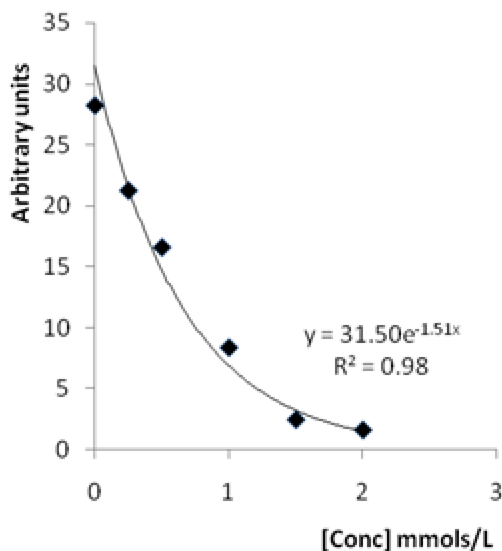


Figure 5. Decrease of the intensity of the spectrum of the spin adduct of methoxy radical with DMPO by increasing the concentration of **EGC**. The best fitting function is an exponential curve with the equation given inside the graphic and in the text.

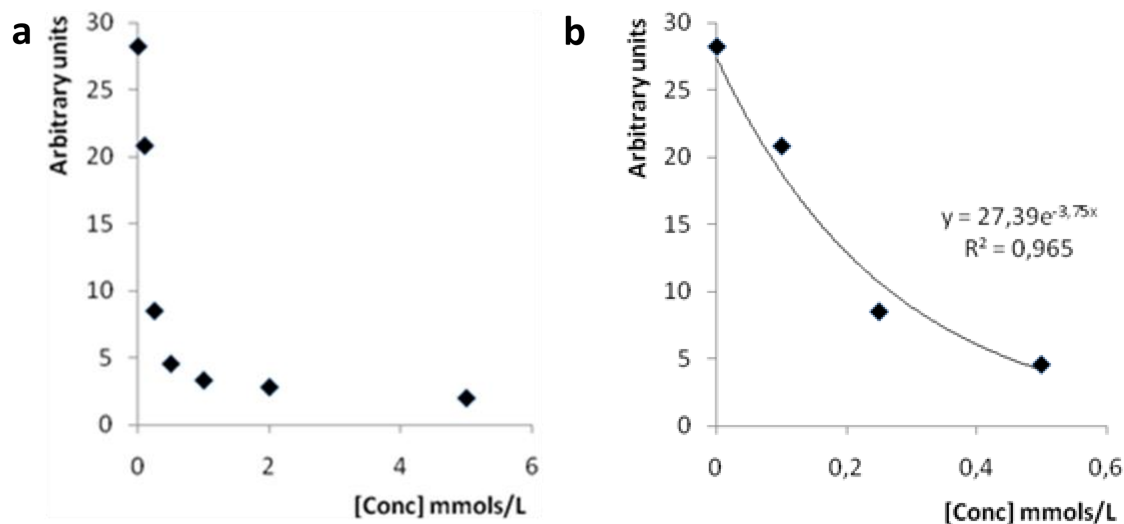


Figure 6. (a) Decrease of the intensity of the spectrum of the spin adduct of methoxy radical with DMPO by increasing concentration of **EGCG**. (b) Detail of the first values of the graph; the best fitting function is an exponential curve with the equation given inside the graphic and in the text.

Graphics for polyphenols EC, ECG, EGC and EGCG are shown in Figures 3-6. In each case, experimental values are fitted to exponential functions inserted in the Figures with good correlation coefficients (R), α being a measure of the antioxidant activity of polyphenols against the methoxy radical.

The graphics corresponding to ECG and EGCG (Figures 4 and 6) show some anomalies. For the exponential regression approach of the results, only low concentration values of polyphenol are well fitted to exponential functions. Both polyphenols show strong antioxidant properties at very low concentrations. High values of concentration give rise to values of integration well above the values that correspond to the exponential functions. This anomalous behavior may indicate the complex processes taking place to remove methoxy radicals within the reaction mixture. This is because the degradation byproducts of polyphenols may in turn act as radical scavengers.

The antioxidant efficiency coefficients of polyphenols relative to that of Ferulic acid are displayed in Table 4. All polyphenols exhibit antioxidant efficiency well above that of the Ferulic acid. The presence of the pyrogallol group and/or gallate group in the molecular

structure of ECG, EGC and EGCG greatly increases the methoxy radical scavenging relative to EC. EGCG with both groups, gallate and pyrogallol, being the most active polyphenol.

Table 4.Values of the Antioxidant Activity Coefficients (α) of Pure Polyphenols EC, ECG, EGC and EGCG Towards Methoxy Radical and its Value Relative to the Ferulic Acid (FA)

Polyphenol	α (mmols/L)-1	α (FA relative)
FA	0.04	1
EC	0.34	8.5
ECG	2.43	60.75
EGC	1.51	37.75
EGCG	3.83	95.75

These results with the spin trap method indicate that EGCG has the greatest radical scavenging activity and it is consistent with those obtained by other more traditional methods, such as Trolox Equivalent Antioxidant Capacity (TEAC) with ABTS radical or Oxygen Radical Antioxidant Capacity (ORAC) with AAPH radical.45,46 The values obtained with the spin trap method show a much greater difference in antioxidant activity among the polyphenols than the other methods, although the order of activity is the same. An advantage of the spin method is that it works with ROS, the true mediators of human diseases, while TEAC and ORAC methods work with "atypical" and more stable radicals.

Table 5 shows the scavenging activity of WT methanolic extract with the different methods. It is remarkable that the correlation is good in all of them. The sensibility for the EPR method is in the middle of the others, expressed as FA equi/g sample (1,3 ± 0,3) and near to the value obtained for the ORAC (1, 75± 0,6 FA equi/g sample) the more sensitive radical scavenging method.

Table 5. Antioxidant Activity of WT Tested by Different Methods with Ferulic Acid (FA) as Standard.

	ORAC		TEAC		EPR		DPPH		FRAP	
WTc	FA equivalents a	FA equi/g sampleb	FA equivalents a	FA equi/g sampleb	FA equivalents a	FA equi/g sampleb	FA equivalents a	FA equi/g sampleb	FA equivalents a	FA equi/g sampleb
542	68.06	1.67	17.46	0.54	2160.00	1.33	755.04	4.64	242.42	2.98
651	48.98	1.81	9.81	0.48	876.67	1.35	1057.11	4.55	154.02	2.84
1627	29.65	1.82	7.91	0.49	706.67	1.30	845.54	4.68	130.11	3.20

a Ferulic Acid equivalents in mg/L. b Ferulic Acid equivalents in g/g of sample. c mg

For the other hand, the results obtained in this paper confirm that the presence of gallate in catechins leads to a higher a radical scavenging activity. And this also corroborates the results of ORAC or TEAC; they work in a similar as the spin trap method.Moreover, there is no correlation between the composition in catechins found in the tea extract and the antioxidant activity values obtained by the spin trap method. That is, a greater proportion of ferulic acid equivalents in g/L would be expected per g/L of tea extract than the value shown in Table 3. This anomaly could be explained because the competition established between catechins finally leads to non-additive of the respective activities. It seems that the main catechin in the composition of WT, EGCG, is the most responsible for the radical scavenging effect, measured by the spin trap method.

3.1.4 Conclusion

In conclusion, we have studied the antioxidant activity of WT extracts and the activity of the most abundant catechins of the WT *versus* methoxy radical, using Ferulic acid as standard. An HPLC analysis of the extracts in MeOH shows the presence of EC, ECG, EGC and EGCG in significant amounts. The source of methoxy radicals has been the reduction of H_2O_2 by FeSO4 (Fenton reaction) in a ratio of 5:1, in MeOH as solvent. The experiments were conducted by electron paramagnetic resonance (EPR) using the spin trap technique in the presence of 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), a good methoxy radical scavenger, and in the presence of varying amounts of antioxidant. In these conditions, the oxidant mixture generates methoxy radical as the only radical species in significant amounts, being identified by EPR as the stable radical adduct DMPO-OCH_3. Therefore, we suggest that the dominant mechanism in the Fenton reaction in these specific conditions is a non-radical mechanism with a two-electron transfer process from Fe(II) to the formation of ferryl ion $[FeIVO]^{2+}$**.** This oxidizing species reacts with MeOH to give methoxy radical and regenerates Fe(II).

The antioxidant activity of the WT extracts and polyphenols has been measured by the decrease of the intensity of the spectral bands of the adduct DMPO-OCH_3 in the EPR with the amount of antioxidant. The activity of WT extracts is slightly higher than that of Ferulic acid as shown in Table 4. In addition, the antioxidant efficiency of all catechins is much

greater than that of the Ferulic acid; the highest being the activity of EGCG and ECG, which contain the gallate group, and the lowest that of EC.

Abbrevations Used

WT, White tea; EC, epicatechin; ECG, epicatechin-3-gallate; EGC, epigallocatechin; EGCG, epigallocatechin-3-gallate; EPR, electron paramagnetic resonance; HO•, hydroxyl radical; O_2•-, superoxide radical; ROS, reactive oxygen species; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; CH_3O•, methoxy radical; MeOH, methanol; min, minutes; h; hour; TEAC, Trolox Equivalent Antioxidant capacity; ORAC; Oxygen Radical Antioxidant Capacity; FA, Ferulic acid; DI, double integral; α, antioxidant activity coefficients.

References

1. Moderno PM, Carvalho M, Silva BM. Recent patents on *Camellia sinensis*: source of health promoting compounds. *Recent Pat. Food. Nutr. Agric.* 2009;1:182–192.

2. Venditti E, Bacchetti T, Tiano, L.; Carloni, P.; Greci, L.; Damiani, E. Hot vs. cold water steeping of different teas: Do they affect antioxidant activity? *Food Chem.* 2010;119:1597–1604.

3. Sharangi AB. Medicinal and therapeutic potentialities of tea (*Camellia sinensis L.*) - A review. *Food Res. Int.* 2009;42:529–535.

4. Rusak G, Komes D, Likic S, Horzic D, Kovac M. Phenolic content and antioxidative capacity of green and white tea extracts depending on extraction conditions and the solvent used. *Food Chem.* 2008;123:46–48.

5. Peres RG, Tonin FG, Tavares MFM, Rodriguez-Amaya DB. Determination of catechins in green tea infusions by reduced flow micellar electrokinetic chromatography. *Food Chem.* 2011;127:651–655.

6. Almajano MP, Carbó R, Jiménez JAL, Gordon MH. Antioxidant and antimicrobial activities of tea infusions. *Food Chem.* 2008;108:55–63.

7. Dias TR, Alves MG, Tomás GD, Socorro S, Silva BM, Oliveira PF. White tea as a promising antioxidant medium additive for sperm storage at room temperature: a comparative study with green tea. *J. Agric. Food Chem.* 2014;62:608–617.

8. Yen, WJ, Chyau CC, Lee CP, Chu HL, Chang LW, Duh PD. Cytoprotective effect of white tea against H_2O_2-induced oxidative stress in vitro. *Food Chem.* 2013;141:4107–4114.

9. Janzen EG. Spin trapping. *Acc. Chem. Res.* 1971;4:31–40.

10. Polovka M. EPR spectroscopy: A tool to characterize stability and antioxidant properties of foods. *J. Food Nutr. Res.* 2006;45, 1–11.

11. Polovka M, Brezová V, Staško A. Antioxidant properties of tea investigated by EPR spectroscopy. *Biophys. Chem.* 2003;106:39–56.

12. Villamena FA, Zweier JL. Detection of reactive oxygen and nitrogen species by EPR spin trapping. *Antioxid. Redox Signal.* 2004;6:619–629.

13. Guo Q, Zhao B, Shen S, Hou J, Hu J, Xin W. ESR study on the structure-antioxidant activity relationship of tea catechins and their epimers. *Biochim. Biophys. Acta.* 1999;1427:13–23.

14. Finkelstein E, Rosen GM, Rauckman EJ. Spin trapping. Kinetics of the reaction of superoxide and hydroxyl radicals with nitrones. 1980; 4994–4999.

15. Thornalley PJ, Vasák M. Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. *Biochim. Biophys. Acta* 1985;827:36–44.

16. Mitsuta K, Mizuta Y, Kohno M, Hiramatsu M, Mori A. The application of ESR spin-trapping technique to the evaluation of SOD-*like* activity of biological substances. *Bull. Chem. Soc. Jpn.* 1990;63:187–191.

17. Naito Y, Yoshikawa T, Tanigawa T, Sakurai K, Yamasaki, K.; Uchida, M.; Kondo, M. Reactions of captopril and epicaptopril with transition metal ions and hydroxyl radicals: an EPR spectroscopy study. *Free Radic. Biol. Med.* 1995;18:117–123.

18. Lauderback CM, Breier AM, Hackett J, Varadarajan S, Goodlett-Mercer J, Butterfield DA. The pyrrolopyrimidine U101033E is a potent free radical scavenger and prevents Fe(II)-induced lipid peroxidation in synaptosomal membranes. *Biochim. Biophys. Acta*. 2000;1501:149–161.

19. Polyakov N. Carotenoids as antioxidants: spin trapping EPR and optical study. *Free Radic. Biol. Med.* 2001;31:43–52.

20. Sueishi Y, Yoshioka C, Olea-Azar C, Reinke LA, Kotake Y. Substituent effect on the rate of the hydroxyl and phenyl radical spin trapping with nitrones. *Bull. Chem. Soc. Jpn.* 2002;75:2043–2047.

21. Kogure K, Tsuchiya K, Abe K, Akasu M, Tamaki T, Fukuzawa K, Terada H. Direct radical scavenging by the bisbenzylisoquinoline alkaloid cepharanthhine. *Biochim. Biophys. Acta.* 2003;1622:1–5.

22. Mesa JA, Chávez S, Fajarí L, Torres JL, Juliá L. A tri(potassium sulfonate) derivative of perchlorotriphenylmethyl radical (PTM) as a stable water soluble radical scavenger of the hydroxyl radical more powerful than 5,5-dimethyl-1-pyrroline-N-oxide. *RSC Adv.* 2013;3:9949-9956.

23. Levêque PP, Godechal Q, Gallez B. EPR spectroscopy and imaging of free radicals in food. *Isr. J. Chem.* 2008;48:19–26.

24. Morsy MA, Khaled MM. Novel EPR characterization of the antioxidant activity of tea leaves. *Spectrochim. Acta. A. Mol. Biomol. Spectrosc.* 2002;58:1271–1277.

25. Biyik R, Tapramaz R. An EPR study on tea: Identification of paramagnetic species, effect of heat and sweeteners. *Spectrochim. Acta. A. Mol. Biomol. Spectrosc.* 2009;74:767–770.

26. Morsy MA. Teas: direct test on quality and antioxidant activity using electron paramagnetic resonance spectroscopy.*Spectroscopy-US.* 2002;16:371–378.

27. Alvarez-Suarez JM, Giampieri F, González-Paramás AM, Damiani E, Astolfi P, Martinez-Sanchez G, Bompadre S, Quiles JL, Santos-Buelga C, Battino M. Phenolics from monofloral honeys protect human erythrocyte membranes against oxidative damage. *Food Chem. Toxicol.* 2012;50:1508–1516.

28. Pérez-Llamas F, González D, Cabrera L, Espinosa C, López JA, Larqué E, Almajano MP, Zamora S. White tea consumption slightly reduces iron absorption but not growth, food efficiency, protein utilization, or calcium, phosphorus, magnesium, and zinc absorption in rats. *J. Physiol. Biochem.* 2011;67:331–337.

29. Almajano MP, Vila I, Gines S. Neuroprotective effects of white tea against oxidative stress-induced toxicity in striatal cells. *Neurotox. Res.* 2011;20: 372–378.

30. Espinosa C, López-Jiménez JÁ, Cabrera L, Larqué E, Almajano MP, Arnao MB, Zamora S, Pérez-Llamas F. Protective effect of white tea extract against acute oxidative injury caused by Adriamycin in different tissues. *Food Chem.* 2012;134:1780–1785.

31. Janzen EG, Liu IP. Radical addition reactions of 5,5-Dimethyl-1-pyrroline-1-oxide. ESR spin trapping with a cyclic nitrone. *J. Magn. Reson.* 1973;9:510–512.

32. Yamazaki I, Piette LH. ESR spin-trapping studies on the reaction of Fe^{2+} ions with H_2O_2-reactive species in oxygen toxicity in biology. *J. Biol. Chem.* 1990;265:13589–13594.

33. Yamazaki I, Piette LH. EPR spin-trapping study on the oxidizing species formed in the reaction of the ferrous ion with hydrogen peroxide. *J. Am. Chem. Soc.* 1991;113:7588–7593.

34. Gozzo F. Radical and non-radical chemistry of the Fenton-like systems in the presence of the organic substrates. *J. Mol. Catal. A Chem.* 2001;171:1–22.

35. Deguillaume L, Leriche M, Chaumerliac N. Impact of radical versus non-radical pathway in the Fenton chemistry on the iron redox cycle in clouds. *Chemosphere.* 2005;60:718–724.

36. Kremer ML. Promotion of the fenton reaction by Cu^{2+} ions: evidence for intermediates. *Int. J. Chem. Kinet.* 2006;38:725–736.

37. Barbusinski K. Fenton Reaction-Controversy concerning the chemistry. *Ecol. Chem. Eng.* 2009;16:347–358.

38. Bataineh H, Pestovsky O, Bakac A. pH-Induced mechanistic changeover from hydroxyl radicals to iron(IV) in the Fenton reaction. *Chem. Sci.* 2012;3:1594.

39. Pestovsky O, Bakac A. Reactivity of aqueous Fe(IV) in hydride atom transfer reactions. *J. Am. Chem. Soc.* 2004;126:13757–13764.

40. Cannon RD. Electron transfer reactions, 1st ed.; Butterworths: London, UK, 1980; pp 315-351.

41. Masarwa M, Cohen H, Meyerstein D, Hickman DL, Bakac A, Espenson JH. Reactions of low valent transition metal complexes with hydrogen peroxide. Are they "Fenton-Like" or not? 1. The case of Cu^{+} aq and Cr^{2+}. *J. Am. Chem. Soc.* 1988;110:4293–4297.

42. Asmus KD, Moeckel H, Henglein A. Pulse radiolytic study of the site of $OH^{\bullet}$ radical attack on aliphatic alcohols in aqueous solution. *J. Phys. Chem.* 1973;77:1218–1221.

43. Jurva U, Wikström HV, Bruins AP. Electrochemically assisted Fenton reaction: reaction of hydroxyl radicals with xenobiotics followed by on-line analysis with high-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 2002;16:1934–1940.

44. Janzen EG, Liu J I P. Radical addition reactions of 5,5-Dimethyl-1-pyrroline-1-oxide. ESR spin trapping with a cyclic nitrone. *J. Magn. Reson.* 1973;9:510–512..

45. Zhang Y, Li Q, Xing H, Lu X, Zhao L, Qu K, Bi K. Evaluation of antioxidant activity of ten compounds in different tea samples by means of an on-line HPLC–DPPH assay. *Food Res. Int.* 2013;53:847–856.

46. Higdon JV, Frei B. Tea catechins and polyphenols: Health effects, metabolism, and antioxidant functions. *Crit. Rev. Food Sci. Nutr.* 2003;43:89–143

3.2. Use of lyophilized and powdered *Gentiana lutea* root in fresh beef patties stored under different atmospheres:

Abstract

Gentiana lutea root is a medicinal herb that contains many active compounds which contribute to physiological effects, and it has recently attracted much attention as a natural source of antioxidants. The aim of this study was to evaluate the effects on the color, pH, microbial activities, sensory quality and resistance to lipid oxidation (through Thiobarbituric method, TBARS) during storage of beef patties containing different concentrations of Gentiana lutea. Fresh beef patties were formulated with 0 – 5 g kg^-1 of *Gentiana lutea* and 0 or 0.5 g kg^-1 of ascorbic acid (AA) and packed in two different atmospheres, Modified Atmosphere 1 (MAP1) and Modified Atmosphere 2 (MAP2), and stored at 4 ± 1^{O}C for 10 days. MAP1 contained 20:80 (v/v) O_2:CO_2 and MAP2 contained 80:20 (v/v) O_2:CO_2. *Gentiana lutea* extracts possessed antioxidant activity measured by Ferric Reducing Antioxidant Power (FRAP) and Oxygen Radical Absorbance Capacity (ORAC) assays. 2 g kg^-1 of lyophilized *Gentiana lutea* was able to inhibit lipid oxidation in both atmospheres ($p<0.05$). Beef patties containing a combination of 2 g kg^-1 *Gentiana lutea* and 0.5 g kg^-1 AA showed significant different in color and lipid oxidation ($p<0.05$). The results from this study manifest the potential of *Gentiana lutea* as a food ingredient in the design of healthier meat commodities.

Keywords:

Gentiana lutea, lipid oxidation, modified atmosphere, antioxidant, beef patties.

3.2.1 Introduction

Nowadays there is growing interest in new sources of natural antioxidants as components of a healthy diet and as a replacement for synthetic antioxidants to maintain food quality. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are commonly used as synthetic antioxidants in food industry. However, their possible toxic effects at high doses, which offset the beneficial effects of extending food shelflife, have raised much concern[1]. Oxidative deterioration commonly occurs in the muscle food and affects the sensory characteristics of meat shelf-life such as colour, flavour, odour and texture. It also provokes the loss of nutritional value and the generation of toxic reaction products such as malonadehyde (MDA)[2,3]. A substantial number of studies have shown that the phenolic compounds exhibit in plant extracts are potent antioxidants which successfully retard lipid oxidation in meat products. Plant derived antioxidants, such as grape seed, rosemary and herb extracts, are of particular interest to food manufacturers because of their ability to delay the oxidation process and improve nutritional quality of meat[3–5].

The antioxidant potential of *Gentiana lutea* to inhibit lipid oxidation of muscle food has not been described yet. Thus, this research has studied the efficacy of lyophilized and powdered *Gentiana lutea* root to prevent deterioration of rounded beef patties during 10 days of refrigerated storage in two different atmospheres: MAP1 and MAP2. MAP1 contained lower oxygen concentration (20:80 (v/v) O_2:CO_2) than MAP2 (80:20 (v/v) O_2:CO_2). However, plant extracts with natural antioxidant activity are not normally able to provide sufficient protection against discoloration of meat products. Therefore, the combination effects of *Gentiana lutea* root products and 0.5 g kg^-1 ascorbic acid (AA) have been studied for their ability to prevent meat discoloration. The measurement of antioxidant effects, pH, microbial analysis, colour and sensory aspects were subjects of the study. Assessment of antioxidant activity of *Gentiana lutea* has been studied by the determination of Total Phenol Content (TPC), Oxygen Radical Absorbance Capacity (ORAC) and Ferric Reducing Antioxidant Power (FRAP) assay.

3.2.2 Materials and Methods

Materials

Dried *Gentiana lutea* was kindly supplied by Manatial de la Salut, a registered herbal company in Barcelona, Spain. AA, reagents and solvents used were analytical grade and obtained from Panreac (Barcelona, Spain) and Sigma Aldrich (Gillingham, England). The lean meat of male bull slaughtered in accordance with approved guidelines of the Animal Ethics Committee (Spain) was supplied by Embutidos La Masia (Barcelona). The meat was collected 7 days after slaughter to allow it to mature and kept at approximately -4 ^{O}C for further treatment.

Extraction of *Gentiana Lutea*

Dried roots of *Gentiana lutea* were cut into 1 x 1 x 1 cm cubes and grounded using a standard kitchen food processor (Moulinex Odacio, Barcelona). Fine grounded *Gentiana lutea* (powdered) was extracted with water and 50:50 (v/v) aqueous ethanol, always in the ratio of 1:10 (w/v). Extraction with aqueous ethanol and water was performed at 4 ± 1 ^{O}C for 24 h in the dark with constant stirring. Each sample was extracted in triplicate and sample extracts were collected for antioxidant assay analysis. The extract solutions of *Gentiana lutea* were recovered by filtration using Whatman Filter paper, 0.45 µm, and excess ethanol was removed under vacuum using a rotary evaporator (Buchi RE111, Switzerland). All extracts were dried in a freeze dryer (Unicryo MC2L +/- 60 ^{O}C, Germany) under vacuum at -60 ± 2 ^{O}C for 3 days in order to remove moisture. Finally, lyophilized *Gentiana lutea* was weighed to determine weight and extraction yield as described by Zhang *et al*.[6]

Determination of the Total Phenolic Content (TPC)

The Folin-Ciocalteu method was used to determine the total phenolic content as reported by Santas *et al*.[7] An appropriate dilution of antioxidant was prepared before adding the Folin-Ciocalteu reagent and sodium carbonate solution. The mixture was finally diluted with miliQ water, shaken and incubated in the dark for 1 hour. Absorbance at 765 nm was measured using a microplate reader (Fluostar Omega, BMG Labtech, Germany) with water as

a blank. Gallic acid was used to prepare a standard calibration line, and the results were expressed as mg of Gallic acid equivalents per g dry weight (mg GAE g^-1 DW).

Determination of Free Radical Scavenging Activity Assays

a. Determination of the Ferric Reducing Antioxidant Power (FRAP)

The FRAP method was introduced by Benzie and Strain[8] and implemented with some modifications. The FRAP solution was prepared in proportions of 10:1:1 of 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3$ respectively. FRAP solution was incubated at 37 ^oC for 20 min before it was mixed with the adequate dilution of the extract. The absorbance was measured at 593 nm using a microplate reader. Results were compared with the Trolox calibration curve, and concentrations were expressed as micromoles of Trolox equivalents per dried weight of sample (µmol of TE g^-1 DW).

b. Determination of the Oxygen Radical Absorbance Capacity (ORAC)

The ORAC value was determined according to Stockham *et al.*[9] with amendment. An appropriate concentration of *Gentiana lutea* extract was mixed with 13 mM of phosphate buffer (incubated at 37 ^oC for 20 min) and 80 mM of fluorescein respectively. 60 mM APPH radical were added after the initial value of fluorescence was recorded and the fluorescence was monitored for 150 minutes using a microplate reader. The net area under the fluorescein decay curve (AUC) was determined and ORAC values were calculated by comparing the AUC to that of Trolox as a standard. All measured data were expressed as µmol of TE g^-1 DW.

Determination of Antioxidant Activity in meat patties

a. Preparation of beef patties

Storage of beef patties in different modified atmospheres were divided into 2 trials using the same samples of *Gentiana lutea* root in powdered and lyophilized form. The first trial (MAP1) was carried out in less oxygen concentration (20:80 (v/v) O_2:CO_2). Fat and joint tissues were trimmed off lean meat (2000 g per trial) and the meat was minced through 8 mm

industrial plates. Then, the minced meat was divided into 5 batches which were mixed with 15 g kg^-1 NaCl and either i) control (no addition), ii) 5 g kg^-1 powdered *Gentiana lutea*, iii) 5 g kg^-1 lyophilized *Gentiana lutea*, iv) 2 g kg^-1 powdered *Gentiana lutea*, v) 2 g kg^-1 lyophilized *Gentiana lutea*. All batches were mixed vigorously for 2 minutes to attain even distribution and moulded into smaller portions (about 20 g each). The samples was stuffed and packed with polystyrene B5-37 (Aerpack) trays and placed in BB4L bags (Cryovac) of low gas permeability (8–12 cm^3 m^-2 per 24 h). The air in the trays was flushed with 20:80 (v/v) O_2:CO_2 by EAP20 mixture (Carburos Metalicos, Barcelona) using packaging machine (ULMA smart 500, Barcelona). Samples were stored in the dark at 4 ± 2 °C for 10 days to measure thiobarbituric acid reactive substances (TBARS), colour, pH and microbial quality. Every measurement was carried out in triplicate each day for 10 days (except for microbiological analysis).

The second trial (MAP2) was prepared using the same procedure as the first trial. Modified atmosphere for MAP2 contained higher oxygen concentration approximately 80:20 (v/v) O_2:CO_2. 7 batch of the meat was mixed with 15 g kg^-1 NaCl and either i) control (no addition), ii) 0.1 g kg^-1 BHT, iii) 0.5 g kg^-1 AA, iv) 0.5 g kg^-1 AA and 2 g kg^-1 lyophilized *Gentiana lutea*, v) 2 g kg^-1 lyophilized *Gentiana lutea,* vi) 2 g kg^-1 powdered *Gentiana lutea* and vii) 1 g kg^-1 lyophilized *Gentiana lutea*. All samples were analyzed by the same procedures used in the first trial.

b. Thiobarbituric acid reacting substances (TBARS)

TBARS method was used to measure the extent of lipid oxidation over the storage period as described by Grau *et al.*[10] Sample (1 g) was weighed in a tube and mixed with 3 g L^-1 aqueous EDTA. Then, the sample was immediately mixed with 5 mL of thiobarbituric acid reagent using an Ultra-Turrax (IKA, Germany) at a speed of 32000 rpm, for 2 min. All procedures were carried out in the dark and all samples were kept in ice. The mixture was incubated at 97 ± 1 °C in hot water for 10 min and shaken for 1 min during the process to form a homogeneous mixture. The liquid sample was recovered by filtration (Whatman Filter paper, 0.45 µm) after the sample was cooled for 10 min. The absorbance value for each sample was measured at 531 nm using a spectrophotometer. The TBARS value was calculated from a malonaldehyde (MDA) standard curve prepared with 1,1,3,3 tetraethoxypropane and analysed by linear regression. All results were reported in mg malonaldehyde per kg of sample (mg MDA kg^-1 sample).

c. Colour measurement

A Chroma meter with measuring head (CR 400 Head, Minolta, Japan) was used to measure the color changes in 3 different parts of the meat: surface, bottom and inner part. The values of a* was measured at 5 different locations of each part. The color was represented by a* values was associated with the redness of the meat. The significant red color of the samples reflected the visual traits of meat such as freshness.

d. Determination of pH and microbiological analysis

The pH value was determined using a micro pH 2001 meter (Crison Instruments, Barcelona). Each pH was measured 3 times for every sample[11]. The microbial content was determined in a Bio–II-A microbiology cabinet to maintain an aseptic environment. Each sample of meat (10 g) was transferred with sterile tweezers to a stomacher bag which contained 10 mL of sterile Ringer´s ¼ solution. A stomacher blender (Seward 80, Spain) was used to homogenize the mixture for 2 min. A series of 10-fold sterile Ringer´s ¼ solution was prepared to dilute homogenized samples (1 mL). Each diluted sample (1 mL) was incubated in triplicate with TCA (Trypticase soy agar, Oxoid, UK) at 15 ^{o}C (for Psychrophiles) and at 30 ^{o}C (for Mesophiles) for 2 to 6 days. The colony count was represented as the logarithm of colony forming units per g (log CFU g^-1).

e. Sensory Analysis

Sensory analysis was performed by a 30 member semi-trained taste panel. A discrimination method (triangle test) was applied to determine the existence of perceptible sensorial differences in appearance and taste between the control (15 g kg^-1 salt) meat patties and the patties with 15 g kg^-1 salt and 2 g kg^-1 lyophilized *Gentiana lutea*. The appearance evaluation was to identify the difference of color and odor of the sample. Samples were presented sliced, on a white plate, at 40 ± 1 ^{o}C. Each panelist was presented with three samples simultaneously, two of which were identical. Each taster was asked to identify the odd sample. Each panelist did the taste test 3 times. The numbers of correct responses given by panelists was determined and the mean value was calculated for each sensorial test. According to Sancho *et al.*[12] for 90 trials, the difference between samples was significant at the appropriate level (i.e. panelists were able to identify the odd sample) if the number of correct answers was 39 ($p<0.05$), 42 ($p<0.01$) and 45 ($p<0.001$).

Statistical Analysis

Statistical analysis was performed using a one-way analysis of variance ANOVA using SPSS 19 (IBM) software program. When statistically significant differences were found, Tukey´s tests were performed and the statistically significance was set at $p<0.05$. The results were presented as mean values ($n \geq 3$).

3.2.3 Results and Discussion

Extraction yield

The extraction was carried out at 4 ± 1 °C to minimise degradation of polyphenols at higher temperatures[13]. The choice of 50:50 (v/v) aqueous ethanol for extraction was based on the results of Nastasijevic *et al.*[14] who reported that this solvent gave an extract of *Gentiana lutea* with the best inhibition activity toward the enzyme myeloperoxidase (MPO) and with the highest antioxidant activity in the DPPH assay; EC_{50} = 20.6 µg mL^-1. The average dried weight of lyophilized obtained after freeze drying was 35 ± 0.4 g L^-1 for aqueous ethanol and 26.8 ± 0.3 g L^-1 for the water extract ($p>0.05$).

Analysis of Total Polyphenols and Free Radical Activity Assays

The phenol content was three times higher after extraction with 50:50 (v/v) aqueous ethanol compared to extraction with water (Table 1). *Gentiana lutea* extracted with 50:50 (v/v) aqueous ethanol also showed higher antioxidant activity than an aqueous extract when assessed by the FRAP and ORAC assays (Table 1). This data is consistent with previous reports which state that antioxidant activity of plant extracts correlates with phenolic content[15] and shows that the yield of phenolic components from herbs is higher with aqueous ethanol rather than water as extraction solvent[16]. Nevertheless, the ORAC result demonstrated ethanol extract gives almost double antioxidant value to scavenge the peroxide radical ($OH^{\bullet}$) generates in assays compared to water extract. Xanthones and its derivatives (such as isogentisin and gentisin) and xanthone glycosides (gentioside and its isomer) are one of the phenolic compounds present in *Gentiana lutea* root expected to be more soluble in aqueous

alcohol. Amarogentin present in *Gentiana lutea* is also a phenolic component expected to have antioxidant properties based on its chemical structure[17].

Table 1: Extraction yield, TPC, FRAP, ORAC of *Gentiana lutea* root extract.

Activity *Gentiana L.*	Extraction solvent	
	H_2O	50:50 (v/v) EtOH:H_2O
[a] Extraction yield (%)	20 ± 0.9%	29.1 ± 0.3%
[b] Total phenolic content (mg GAE g^-1 DW)	3.79 ± 1.7	12.03 ± 1.8
[c] FRAP (µmol of TE g^-1 DW)	10.34 ± 1.5	15.89 ± 0.5
[d] ORAC (µmol of TE g^-1 DW)	26.12 ± 2.3	58.9 ± 1.8

[a.] Extraction yield (%) is calculated according to the method of Zhang *et al.*[16]. [b.] Folin-Ciocalteu measurement of phenolic content equivalent in mg GAE g^-1 DW. [c,d] Antioxidant activity of *Gentiana lutea* determined by FRAP and ORAC assays in µmol of TE g^-1 DW. Mean value n=3 and the standard deviation for each assay is less than 5%.

Estimation of polyphenol content in TPC is pertinent to the FRAP value which showed the phenolic compound in the extract act as electron donator to ferric tripyridyltriazine complex (Fe(III)-TPTZ) to ferrous complex (FE(II)-TPTZ). Previously reported antioxidant assays on extracts from *Gentiana lutea* are limited to DPPH scavenging assay. However, recent finding had demonstrated the antioxidant potency of *Gentiana lutea* extract with water and methanol aqueous on Trolox Equivalent Antioxidant Capacity (TEAC) and superoxide activity assays[18]. Nevertheless, this is the first report of the antioxidant activity of extracts from *Gentiana lutea* roots assessed using the FRAP and ORAC methods.

Antioxidant Effect in meat patties

a. Colour evolution

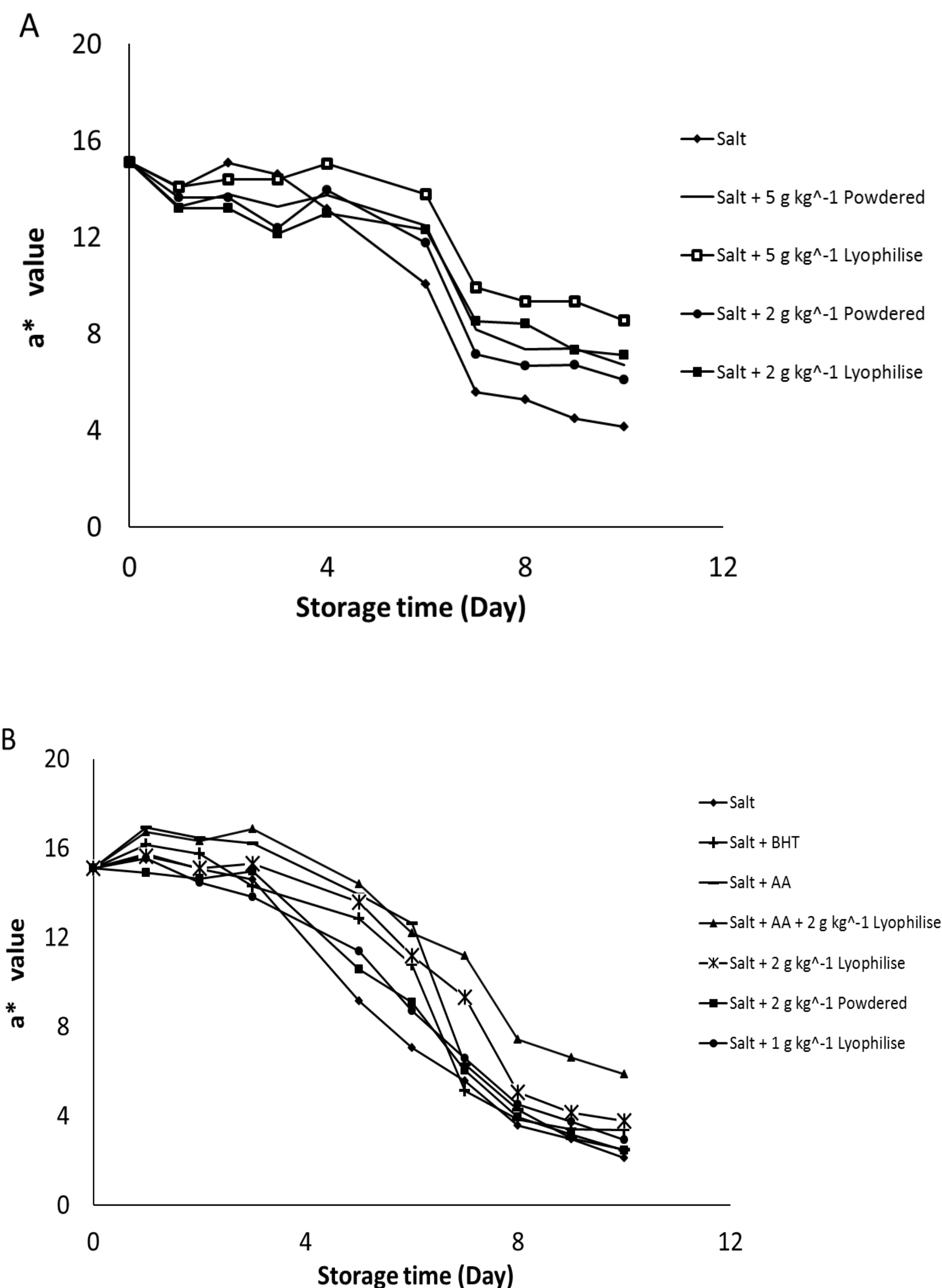


Figure 1: Changes in redness values (a*) of control and samples containing different concentrations (w/w) of *Gentiana lutea* in (A) MAP1 atmosphere and (B) MAP2 during 10 days storage at 4 ± 1^{O}C without light. Each sample was stored and measured in triplicate and the average standard deviation was less than 5%. Concentration of salt (15 g kg^-1), BHT (0.1 g kg^-1) and AA (0.5 g kg^-1).

Many consumers scrutinize meat freshness by its visual redness, thus the a* value justified by the evidence that pigment change and lipid deterioration in muscle foods are closely interrelated[19]. Meat was stored with added salt under MAP1 and MAP2 atmosphere and the red color (a* value) started to decrease after an initial lag phase (Figure 1 A and B). Addition of salt made red color of samples more intense (a* value) than unsalted meat. The red color of the meat was enhanced by the addition of NaCl and lactates with immediate effect as reported previously[2]. The mean a* value of the beef patties was measured as 15.12 ± 1.57 immediately after mixing.

Figure 1A and Figure 1B shows all samples suffered detrimental color changes throughout the storage time. It has been previously reported that high oxygen concentrations enhance the bright-red colour of fresh meat due low concentrations oxygen accelerate the oxidation of myoglobin to metmyoglobin which turns the colour brown[20]. However, from our finding here, there are no significant different of the red value of control samples between trials which may due to influences of salt adding in the samples[21]. Overall, samples containing lyophilized *Gentiana lutea* showed less color change than powdered *Gentiana lutea* samples in both modified atmospheres. There are no significant reduction of color showed by all samples in MAP1 after 7 days ($p>0.05$). The sample containing higher concentration of lyophilized *Gentiana lutea* (5 g kg^-1) showed significantly less loss of redness ($p<0.05$) compared to the control after 4 days in MAP1. However, 2 g kg^-1 *Gentiana lutea* (powdered and lyophilized) in both atmospheres experienced deterioration of red color compared to control samples ($p<0.05$) during 10 days storages. All samples showed a* values in the range of 2 – 6 after 10 days storage in MAP2 condition. In higher oxygen packaging, meat treated with AA plus 2 g kg^-1 *Gentiana lutea* able to maintain and prolong the attractive red color during storages compared to control and synthetic antioxidants (BHT and AA) ($p<0.05$). Whereas, positive control samples (BHT and AA) sustained a*value until 6 days storages before the color value declined rapidly. Many authors claim that adding ascorbic acid to natural antioxidant is beneficial to control the oxidation and the redness of beef patties[22–24]. It act as a reducing agent which inhibits the myoglobin oxidation and brown color development in beef.[23] Therefore, a combination of 2 g kg^-1 lyophilized *Gentiana lutea* with 0.5 g kg^-1 AA showed synergic effect capable of maintaining the color stability during 10 days of storages in MAP2.

b. TBARS values

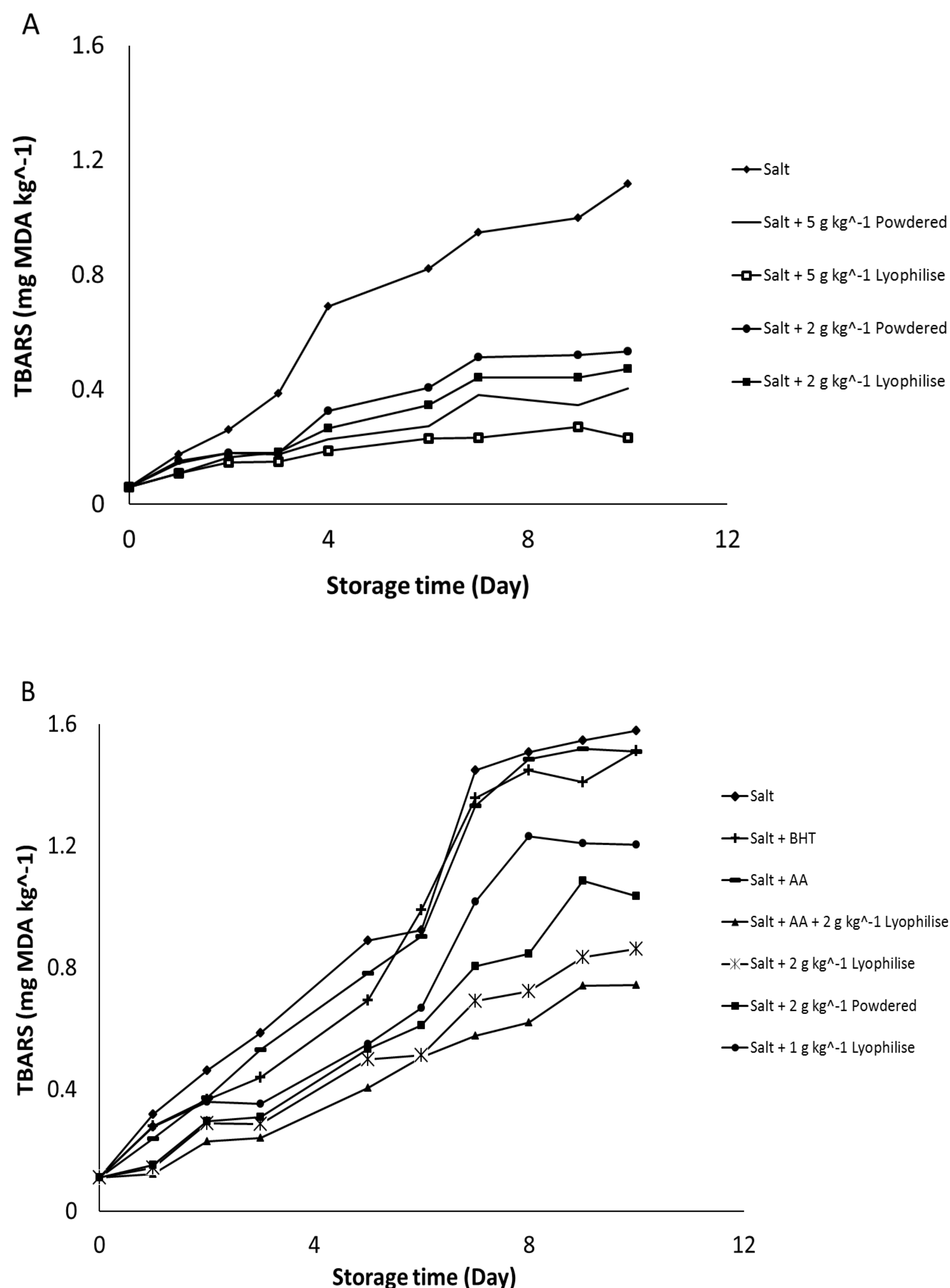


Figure 2: Changes in TBARS values (mg MDA kg^-1) of control and samples containing different concentrations of *Gentiana lutea* in (A) MAP1 and (B) MAP2 atmosphere during 10 days storage at 4 ± 1^{O}C without light. Each sample was stored and measured in triplicate and the average standard deviation for each sample was less than 5%. Concentration of salt (15 g kg^-1), BHT (0.1 g kg^-1) and AA (0.5 g kg^-1).

The research was carried out principally to assess the effect of the *Gentiana lutea* concentration (lyophilized and powdered) on meat properties. Figure 2A and B below shows the effect of the modification of the atmosphere on beef patties containing antioxidants in two oxygen concentrations packaging; MAP1 and MAP2. Overall, samples containing lyophilized *Gentiana lutea* showed less oxidation compared to powdered *Gentiana lutea* samples in both modified atmospheres (Figure 2A and 2B).

The TBARS values of samples containing 2 g kg^-1 lyophilized *Gentiana lutea* in MAP1 and MAP2 were significantly lower than the control ($p<0.05$) throughout the storage period. The TBARS values (control and 2 g kg^-1 *Gentiana lutea*) in MAP1 showed less oxidation compared to the samples stored under MAP2 atmosphere. Our results were in accordance with those of Martinez *et al.*[36], who reported that the higher oxygen concentration resulted in higher TBARS values and an increased oxidation rate in muscle foods

The first trial was carried out in less oxygen packaging (MAP1) between 2 - 5 g kg^-1 of *Gentiana lutea.* The initial concentration proposed during the first trial was approximate from our pervious study in the emulsion system[18]. TBARS value for all samples shown in Figure 2A started around 0.1 mg MDA kg^-1 sample and remained less than 0.5 mg MDA kg^-1 sample during 3 days. Then, slow increases of the values were observed due to the low oxygen concentration. All samples treated with *Gentiana lutea* were less oxidized than the control during storages ($p<0.05$). The highest concentration of *Gentiana lutea* (5 g kg^-1) showed the lowest TBARS value during 10 days storages. Control was the only sample reached value of 1.0 mg MDA kg^-1 during the final day of the storages which the sample was considered as rancid. The acceptable limits of TBARS value in fat product was set at 1.0 mg MDA kg^-1 as a guarantee of the product quality[25].

TBARS results obtained in MAP1 showed a minimum concentration 2 g kg^-1 *Gentiana lutea* was successfully inhibit lipid oxidation in beef patties; hence, following trial was carried out at higher oxygen concentration (MAP2). High oxygen in MAP mixtures of 60-80% O_2 and 20-40% CO_2 are commonly used in beef product packaging for retail display in the market. The trial was performed with 1 - 2 g kg^-1 *Gentiana lutea* and addition of 0.5 g kg^-1 AA was to preserve the redness changes during storages. The TBARS values for samples treated with 2 g kg^-1 lyophilized *Gentiana lutea* (with or without AA) were significantly lower throughout storages ($p<0.05$) when compared to all control samples (Figure 2B). The TBARS values of samples containing 1 g kg^-1 lyophilized *Gentiana lutea*

were not significantly different ($p>0.05$) whereas 2 g kg^-1 powdered showed nearly different from all controls during storages ($p<0.1$). Meat samples treated with *Gentiana lutea* with or without AA reached lower TBARS values (less than 0.8 mg MDA kg^-1 sample) after the first 6 days ($p<0.05$). The effect of adding AA plus *Gentiana lutea* antioxidant to the beef patties gave the lowest TBARS values at the final day, which was 0.74 mg MDA kg^-1 under MAP2 condition. These results are consistent with other studies and suggest that antioxidants, such as rosemary and AA in combination, exerted a synergistic effect in preventing lipid oxidation in ground meat[22–24]. The oxidation degradation of the meat patties was more reduced at higher concentration of *Gentiana lutea,* as shown by comparison of the rates for 1 g kg^-1 and 2 g kg^-1 lyophilized addition. During the final phases of storage the all samples except 2 g kg^-1 lyophilized *Gentiana lutea* (with or without AA) reached values greater than 1.0 mg MDA kg^-1 sample. For the 10 days storage period in MAP2, samples treated with 2 g kg^-1 lyophilized *Gentiana lutea* (with or without AA) did not reached limit level of TBARS that justified the meat as a rancid.

Positive controls in MAP2 displayed lower TBARS values during the first 6 days and subsequently increased rapidly to reach similar values to the control after 7 days ($p>0.05$). Level of concentration BHT and AA used in the experiment are approved by FDA and specified under code of Federal Regulations (CFR). BHT are relatively effective at lower concentration and become pro-oxidant in high level, while AA possessed antioxidant properties and can also act as pro-oxidant depending on the concentration, metal ions and tocopherol content[26]. Moreover, there are few reports that have found that ascorbic acid alone is inactive to prevent lipid oxidation in muscle foods[22,23].

The active properties of *Gentiana lutea* have been reported several times by Aberham *et al.*[27,28] with commercial and fresh plants. The root compounds consist in the family of iridoids, scoiridoids and xanthones which could be developed as antioxidant agents and radical scavengers and may contribute to the decrease of lipid oxidation[29]. These compounds have been described to have antioxidant properties and are capable of scavenging free radicals and removing the superoxide radicals naturally produced in cells[30]. Recent studies are focused on evaluating the potential of natural plants to improve the nutritional properties, to reduce lipid oxidation and to extend the shelf life of meat products. The protective effect of adding natural plants against lipid oxidation in meat is due to the presence of phenolic compounds. The antioxidant activity of phenolic compounds is closely related to the hydroxyl group linked to the aromatic ring which is capable of donating hydrogen atoms and

neutralizing free radicals. This mechanism prevents further degradation of reactive oxidizing species such as MDA, which can be measured by the TBARS method[31].

However, the effectiveness of some antioxidants can increase with concentration, but the effect only takes place up to a certain concentration. The active ingredients that exist in the plants can also act as pro-oxidants according to their chemical properties, environmental condition and their interaction with lipids[32]. Many papers have reported that natural plants work as antioxidants efficiently, but it is most important to identify the minimum concentration to reduce lipid oxidation significantly throughout the storage time. Adding the minimum amount of natural plant can not only delay lipid oxidation but also may avoid some changes in sensorial quality and flavor which limit their application in meat. Modified atmosphere packaging (MAP) is one of control test system keeping much attention from researchers to evaluate the effectiveness of functional ingredients such as rosemary, thymol, and natural seed extract in meat products[5,33,34]. MAP2 with 80:20 (v/v) O_2:CO_2 was reported to be the most suitable mixture[35] and effective antimicrobial factor[36] for meat products.

Thus, analysis of our data showed that 2 g kg^-1 lyophilized *Gentiana lutea* was adequate to significantly inhibit deterioration in beef patties ($p<0.05$) in high and low oxygen concentration packaging compared to control. Moreover, a mixture of 2 g kg^-1 lyophilized *Gentiana lutea* with 0.5 g kg^-1 AA exhibited synergic effects on delaying the oxidation rates and improving color of the meat in higher concentration atmosphere. The study confirmed the potential of edible *Gentiana lutea* for preventing oxidation of muscle foods.

c. pH and Microbial analysis

Treatment of the meat with the different *Gentiana lutea* concentrations or combinations of *Gentiana lutea* antioxidants with salts did not alter the pH. The mean pH value measured at the first day was 5.96 ± 0.2 and the values increased less that 0.2 pH units ($p>0.05$) throughout 10 days storage. Slight changes in pH values during storage were also reported by Franco *et al*.[37] They also reported that the pH value was not affected by different packaging atmospheres and did not increase with storage time (80:20 O_2:CO_2 v/v vs. vacuum packaged).

All samples started at 5 log CFU g^-1 microbial count ($p>0.05$), a common value for fresh meat products, following with sample preparation such as mincing and grinding. There were no significant differences in bacterial counts for all samples during 14 days of

incubation; at 10°C (*Psychrophiles*) and 30°C (*Mesophile*) ($p>0.05$). Bitter agents of *Gentiana lutea* (secoiriodoid-glycosides) have been described perviously as anti-fungal and anti-bacterial[38,39]. In the findings here explained, lyophilized and powdered *Gentiana lutea* (1 – 5 g kg^-1) did not display any significant antimicrobial activity against *Mesophilic* and *Psychrophilic* bacteria. Weckesser *et al.*[40] reported that there was no activity of *Gentiana lutea* against several gram positive bacteria and yeasts except marginally against *S. pyogenes*.

d. Sensory Analysis

Minor changes during storage without effects on the taste and appearance of the meat containing added natural antioxidants are required for commercial success in the food industry. A sensory testing result by a triangular test was carried out to assess if the panelists were able to distinguish between control and modified patties. Results confirmed that color and odor did not show statistical differences between control and modified products for most of the panel members. 90% of the panelists indicate that that the appearances (color and odor) of the patties were very similar between control and modified samples. In relation to taste, 70% of the panelists found that there were no significant differences between the control and the addition of 2 g kg^-1 lyophilized *Gentiana lutea* in the patties. These results determined that products with 2 g kg^-1 lyophilized *Gentiana lutea* substitution were viable from the appearance of the patties. However, greater number of the respondents could correctly identify the modified patties on the tasting analysis due to some bitter tasting present in them. The trial was carried out with the minimum concentration, 2 g kg^-1 lyophilized *Gentiana lutea,* to prove its potential use in products without adversely affecting the sensory characteristics as well as positive effects in inhibiting the oxidation of lipids in muscle foods.

3.2.4 Conclusions

Addition of 2 g kg^-1 lyophilized *Gentiana lutea* effectively controlled lipid oxidation in high and low oxygen concentration atmospheres (MAP1 and MAP2) in 4 ± 1 ^{O}C throughout 10 days storage. Adding 2 g kg^-1 *Gentiana lutea* alone did not influence the meat redness, but in combination with 0.5 g kg^-1 ascorbic acid, it showed synergic effect that reduced loss of redness and TBARS values in the meat patties. Sensory analysis indicates the potential of using 2 g kg^-1 *Gentiana lutea* without significantly altering the appearance

and the taste of modified patties. Thus, this study confirmed *Gentiana lutea* roots as a source of edible natural antioxidants that can be used by the food industry for addition to meat products.

References

1. Tokusoglu O and Basmacioglu H. Alternative antioxidants for preservation. *Fleischwirtsch International.* 2004;2: 92-94

2. Tan W and Shelef LA. Effects of sodium chloride and lactates on chemical and microbiological changes in refrigerated and frozen fresh ground pork. *Meat Sci.* 2002;62:27-32.

3. Estevez M, Ventanas S and Cava R. Protein oxidation in frankfurters with increasing levels of added rosemary essential oil: Effect on color and texture deterioration. *J Food Sci.* 2005;70: 427-432.

4. Jongberg S, Torngren MA, Gunvig A, Skibsted LH and Lund MN, Effect of green tea or rosemary extract on protein oxidation in Bologna type sausages prepared from oxidatively stressed pork. *Meat Sci.* 2013;93:538–546.

5. Balijagic J, Jankovic T, Zdunic G, Boskovic J, Savikin K, Godevac D, Stanojkovic T, Jovancevic M and Menkovic N. Chemical Profile, Radical Scavenging and Cytotoxic Activity of Yellow Gentian Leaves (Genitaneae luteae folium) Grown in Northern Regions of Montenegro. *Nat Prod Commun.* 2012;7: 1487-1490.

6. Mathew A, Taranalli AD and Torgal SS. Evaluation of anti-inflammatory and wound healing activity of Gentiana lutea rhizome extracts in animals. *Pharm Biol,* 2004;42:8-12.

7. Ozturk N, Korkmaz S, Ozturk Y and Baser KHC. Effects of gentiopicroside, sweroside and swertiamarine, secoiridoids from gentian (Gentiana lutea ssp symphyandra), on cultured chicken embryonic fibroblasts. *Planta Med.* 2006;72:289-294.

8. Nastasijevic B, Lazarevic-Pasti T, Dimitrijevic-Brankovic S, Pasti I, Vujacic A, Joksic G and Vasic V. Inhibition of myeloperoxidase and antioxidative activity of Gentiana lutea extracts. *J Pharm Biomed Anal.* 2012;66:191-196.

9. Aberham A, Pieri V, Croom EM, Ellmerer E and Stuppner H. Analysis of iridoids, secoiridoids and xanthones in Centaurium erythraea, Frasera caroliniensis and Gentiana lutea using LC-MS and RP-HPLC. *J Pharm Biomed Anal.* 2011;54:517-525.

10. Isiguro K, Yamaki M, Takagi S, Ikeda Y, Kawakami K, Ito K and Nose T. Studies on Iridoid-Related Compounds .4. Antitumor-Activity of Iridoid Aglycones. *Chem Pharm Bull.* 1986;34:2375-2379.

11. Vandersluis WG, Vandernat JM and Labadie RP. Secoiridoids and Xanthones in the Genus Centaurium .5. Thin-Layer Chromatographic Bioassay of Iridoid and Secoiridoid Glucosides with a Fungitoxic Aglucone Moiety using Beta-Glucosidase and the Fungus Penicillium-Expansum as a Test Organis. *J Chromatogr.* 1983;259:522-526.

12. Recio MD, Giner RM, Manez S and Rios JL. Structural Considerations on the Iridoids as Antiinflammatory Agents. *Planta Med.* 1994;60:232-234.

13. Li JC, Feng L, Sun BH, Ikeda T and Nohara T. Hepatoprotective activity of the constituents in Swertia pseudochinensis. *Biol Pharm Bull.* 2005;28:534-7.

14. Hajimehdipoor H, Sadeghi Z, Elmi S, Elmi A, Ghazi-Khansari M, Amanzadeh Y and Sadat-Ebrahimi SE. Protective effects of Swertia longifolia Boiss. and its active compound, swerchirin, on paracetamol-induced hepatotoxicity in mice. *J Pharm Pharmacol.* 2006;58:277-80.

15. Ashida S, Noguchi SF and Suzuki T, Antioxidative Components, Xanthone Derivatives, in Swertia-Japonica Makino. *J. Am. Oil Chem. Soc.* 1994;71:1095-1099.

16. Gonda R, Takeda T and Akiyama T. Studies on the constituents of Anaxagorea luzonensis A. Gray. *Chem Pharm Bull.* 2000;48:1219-22.

17. Chen SX, Wan M and Loh BN. Active constituents against HIV-1 protease from Garcinia mangostana. *Planta Med*,1996;62:381-2.

18. Bajpai MB, Asthana RK, Sharma NK, Chatterjee SK and Mukherjee SK. Hypoglycemic Effect of Swerchirin from the Hexane Fraction of Swertia-Chirayita. *Planta Med.* 1991;57:102-4.

19. Basnet P, Kadota S, Shimizu M and Namba T. Bellidifolin - a Potent Hypoglycemic Agent in Streptozotocin (Stz)-Induced Diabetic Rats from Swertia-Japonica. *Planta Med*. 1994;60:507-11.

20. Schmieder A, Schwaiger S, Csordas A, Backovic A, Messner B, Wick G, Stuppner H and Bernhard D. Isogentisin - A novel compound for the prevention of smoking-caused endothelial injury. *Atherosclerosis.* 2007;194:317-325.

21. Suzuki O, Katsumata Y, Oya M, Chari VM, Klapfenberger R, Wagner H and Hostettmann K. Inhibition of Type-a and Type-B Monoamine-Oxidase by Isogentisin and its 3-O-Glucoside. *Planta Med.* 1980;39:19-32.

22. Zhang S, Bi H and Liu C, Extraction of bio-active components from Rhodiola sachalinensis under ultrahigh hydrostatic pressure. *Separation and Purification Technology.* 2007;57:277-282.

23. Santas J, Carbo R, Gordon MH and Almajano MP. Comparison of the antioxidant activity of two Spanish onion varieties. *Food Chem.* 2008;107:1210-1216.

24. Benzie I and Strain J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal Biochem.* 1996;239:70-76.

25. Stockham K, Paimin R, Orbell JD, Adorno P and Buddhadasa S. Modes of handling Oxygen Radical Absorbance Capacity (ORAC) data and reporting values in product labelling. *J Food Compos Anal.* 2011;24:686-691.

26. Grau A, Guardiola F, Boatella J, Barroeta A and Codony R. Measurement of 2-thiobarbituric acid values in dark chicken meat through derivative spectrophotometry: Influence of various parameters. *J Agric Food Chem.* 2000;48:1155-1159.

27. Banon S, Mendez L and Almela E. Effects of dietary rosemary extract on lamb spoilage under retail display conditions. *Meat Sci.* 2012;90:579-83.

28. Sancho J, Bota E and De Castro J J. Sensorial Analysis de los alimentos, in *Introduccion al análisis sensorial de los alimentos*. Ed. by Universidad de Barcelona, Barcelona, 1999, pp. 219.

29. Gorinstein S, Leontowicz H, Leontowicz M, Namiesnik J, Najman K, Drzewieck J, Cvikrova M., Martincova O, Katrich E and Trakhtenberg S. Comparison of the main bioactive compounds and antioxidant activities in garlic and white and red onions after treatment protocols. *J Agric Food Chem.* 2008;56:4418-4426.

30. Carnat A, Fraisse D, Carnat AP, Felgines C, Chaud D and Lamaison JL, Influence of drying mode on iridoid bitter constituent levels in gentian root. *J Sci Food Agric* 2005;85: 598-602.

31. Shan B, Cai YZ, Sun M and Corke H. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *J Agric Food Chem.* 2005;53:7749-7759.

32. Wang HF, Provan GJ and Helliwell K. Determination of rosmarinic acid and caffeic acid in aromatic herbs by HPLC. *Food Chem.* 2004;87:307-11.

33. Arino A, Arberas I, Leiton M, deRenobales M and Dominguez J. The extraction of yellow gentian root (Gentiana lutea L.). *Z Lebensm Unters Forsch A-Food Res Technol.* 1997;205:295-299.

34. Deighton N, Brennan R, Finn C and Davies HV. Antioxidant properties of domesticated and wild Rubus spices. *J Sci Food Agr.* 2000;80:1307–1313.

35. Gazzani G, Papetti A, Massolini G and Daglia M. Antioxidative and pro-oxidant activity of water soluble components of some common diet vegetables and the effect of thermal treatment. *J Food Chem.* 1998;6:4118–4122.

36. Sanchez-Escalante A, Djenane D, Torrescano G, Beltran JA and Roncales P. Antioxidant action of borage, rosemary, oregano, and ascorbic acid in beef patties packaged in modified atmosphere. *J Food Sci.* 2003;68:339-344.

37. Martinez L, Djenane D, Cilla I, Beltran JA and Roncales P. Effect of varying oxygen concentrations on the shelf-life of fresh pork sausages packaged in modified atmosphere. *Food Chem.* 2006;94: 219-255.

38. O'Grady MN, Monahan FJ and Brunton N. Oxymyoglobin oxidation and lipid oxidation in bovine muscle – mechanistic studies. *J Food Sci*. 2001;66:386–392.

39. Hayes JE, Stepanyan V, Allen P, O'Grady MN and Kerry JP. Effect of lutein, sesamol, ellagic acid and olive leaf extract on the quality and shelf-life stability of packaged raw minced beef patties. *Meat Sci.* 2010;84:613–620.

40. Kennedy C, Buckley DJ and Kerry JP. Display life of sheep meats retail packaged under atmospheres of various volumes and compositions. *Meat Sci.* 2004;68:649-658.

41. Kim SJ, Min SC, Shin HJ, Lee YJ, Cho AR, Kim SY and Han J. Evaluation of the antioxidant activities and nutritional properties of ten edible plant extract and their application to fresh ground beef. *Meat Sci.* 2013;93:715-722.

42. Doménech-Asensi G, García-Alonso FJ, Martínez E, Santaella M, Martín-Pozuelo G, Bravo S, Periago MJ. Effect of the addition of tomato paste on the nutritional and sensory properties of mortadella. *Meat Sci,* 2013;93:213-219.

43. Kušar A, Zupančič A, Šentjurc M and Baričevič D. Free radical scavenging activities of yellow gentian (Gentiana lutea L.) measured by electron spin resonance. *Hum Exp Toxicol.* 2006;25:599-604.

44. Oliveira TLC, Carvalho SM, Araújo Soares R, Andrade MA, Graças Cardoso M, Ramos EM and Piccoli RH. Antioxidant effects of Satureja montata L. essential oil on TBARS and color of mortadella-type sausages formulated with different levels of sodium nitrite. *LWT Food Sci. Technol.* 2011;45:204-212.

45. Lara MS, Gutierrez JI, Timon M and Andres AI. Evaluation of two natural extract (Rosmarinus officinalis L. and Meissa officinalis L.) as antioxidants in cooked pork patties packed in MAP. *Meat Sci* 2011;88:481-488.

46. Hayes JE, Stepanyan V, Allen P, O'Grady MN and Kerry JP. Evaluation of the effects of selected plant-derived nutraceuticals on the quality and shelf-life stability of raw and cooked pork sausages. *LWT Food Sci. Technol.* 2011;44:164-172.

47. Alp E and Aksu MI. Effects of water extract of Urtica dioica L. and modified atmosphere packaging on the shelf life of ground beef. *Meat Sci.* 2010;86:468–473.

48. Sampaio GR, Saldanha T, Soares RAM and Torres EAFS. Effect of natural antioxidant combinations on lipid oxidation in cooked chicken meat during refrigerated storage. *Food Chem.* 2012;135:1383–1390.

49. Lin Y, Huang M, Zhou G, Zou Y and Xu Xinglian. Prooxidant Effects of the Combination of Green Tea Extract and Sodium Nitrite for Accelerating Lipolysis and Lipid Oxidation in Pepperoni during Storage. *J Food Sci.* 2012;45:204–212.

50. Kim YH, Huff-Lonergan E, Sebranek JG and Lonergan SM. High-oxygen modified atmosphere packaging system induces lipid and myoglobin oxidation and protein polymerization. *Meat Sci.* 2010;85:759-767.

51. Franco D, Gonzalez L, Bispo E, Latorre A, Moreno T, Sineiro J, Sanchez M and Nunez JM. Effects of calf diet, antioxidants, packaging type and storage time on beef steak storage. *Meat Sci.* 2012;90:871-880.

52. Guerin JC and Reveillere HP. Antifungal Activity of Plant-Extracts used in Therapy .2. Study of 40 Plant-Extracts Against 9 Fungi Species. *Ann Pharm Fr.* 1985;43:77-81.

53. Weckesser S, Engel K, Simon-Haarhaus B, Wittmer A, Pelz K and Schempp CM. Screening of plant extracts for antimicrobial activity against bacteria and yeasts with dermatological relevance. *Phytomedicine.* 2007;14:508-516.

3.3. Screening of Antioxidant Activity of *Gentiana Lutea* Root and Its Application in Oil-in-Water Emulsions

Abstract

Gentiana Lutea root is a medicinal herb, traditionally used as a bitter tonic in gastrointestinal ailments for improving the digestive system. The active principles of *Gentiana Lutea* were found to be secoiridoid bitter compounds as well as many other active compounds causing the pharmacological effects. No study to date has yet determined the potential of *Gentiana Lutea* antioxidant activity on lipid oxidation. Thus, the aim of this study was to evaluate the effects of an extract of *Gentiana Lutea* on lipid oxidation during storage of an emulsion. *Gentiana Lutea* extracts showed excellent antioxidant activity measured by DPPH scavenging assay and Trolox equivalent antioxidant capacity (TEAC) assays. An amount of 0.5% w/w *Gentiana Lutea* lyophilise was able to inhibit lipid oxidation throughout storage ($p < 0.05$). A mixture of *Gentiana Lutea* with 0.1% (w/w) BSA showed a good synergic effect and better antioxidant activity in the emulsion. Quantitative results of HPLC showed that *Gentiana Lutea* contained secoiridoid-glycosides (gentiopiocroside and sweroside) and post column analysis displayed radical scavenging activity of *Gentiana Lutea* extract towards the ABTS radical. The results from this study highlight the potential of *Gentiana Lutea* as a food ingredient in the design of healthier food commodities.

Keywords:

Gentiana Lutea; lipid oxidation; antioxidant; HPLC

3.3.1 Introduction

Gentiana Lutea root also known as Yellow Gentian, has over 300 species and is widely distributed in North America, Europe, Asia and some parts of South America[1]. The plant root was traditionally use as a medicinal plant to stimulate appetite and improve digestion[2]. Furthermore, the root is also well known for its bitter properties due to the existence of secoiridoid-glycosides (e.g., swertiamarin, gentiopicroside, amarogentin and sweroside)[3]. Many researchers have discovered the benefits of bitter tasting secoiridoid-glycoside through extensive pharmacological studies. These constituents are claimed to have many biological effects such as anti-apoptotic[4], anti-cancer[5], anti-fungi[6], anti-bacterial[7], anti-inflammatory[8], and hepatoprotective[9]. It has been reported recently by Nastasijevic et al. (2012) that the *Gentiana Lutea* extracts have potential to inhibit myeloperoxidase (MPO) activity which contributes to many disorders such as cardiovascular, inflammatory, neurodegenerative and immune-mediated diseases[10].

However, not only are the secoiridoids relevant for the plant pharmacological action, there are many active compounds in *Gentiana Lutea* that also have relevant effects such as iridoidloganic acid, xanthone (e.g., gentisin and isogentisin) and xanthone glycosides (gentioside and its isomer)[3]. Iridoidloganic acid has shown potent activity as an anti-inflammatory and a number of studies demonstrated that xanthones and its derivatives have wide-ranging biological activities such as anti-inflammatory, anti-hepatotoxic, anti-tumor and anti-microbial[11]. Furthermore, xanthone, and its derivatives, are phenolic compounds with antioxidant properties which have attracted much attention recently[12]. There are few reports that have investigated the radical scavenging activities of secoiridoid-glycosides using DPPH assay[7,13], although some authors have measured total antioxidant capacity of *Gentiana Lutea* but without sufficient assessment of individual antioxidants[10,14]. However, the observation of antioxidant activity of *Gentiana Lutea* extract towards lipid oxidation has not been fully determined.

Lipid oxidation in high fat-containing food is a major cause of shelf life deterioration such as in meat products and emulsions. The oxidation process causes unsavoury alteration of flavor, texture, shelf life, appearance, and nutritional qualities[15].Oil-in-water emulsion (o/w), is a food model which is highly susceptible to oxidation, besides it has also become one of the effective models to evaluate the antioxidant activity of natural plants towards lipids[16]. The first phase of lipid oxidation starts with the formation of unstable free radicals and

hydroperoxides with further decomposition to secondary products like ketones, aldehydes, alcohols and acids[17]. The formation of peroxides in primary oxidation can be measured using peroxide value (PV) assay. Secondary oxidation can be measured by thiobarbituric acid reacting substances (TBARS), specifically aldehydes, and also leads to unpleasant taste, aroma and quality traits of the products.

Thus, our goal was to evaluate the potential antioxidant activity of *Gentiana Lutea* by different methods: (1) *in vitro* with such radicals as $ABTS^{\bullet+}$, DPPH and enzymatic activity and (2) in o/w emulsion.

3.3.2 Materials and Methods

Materials

Commercially dried *Gentiana Lutea* was kindly supplied by Manatial de la Salut (Barcelona, Spain), a registered herbal company. Reagents used were: thiobarbituric acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, methanol, hydrogen chloride, aluminium oxide, ferrous chloride, anhydrous sodium carbonate, ethanol 96%, Phosphate Buffer Solution (PBS) and ammonium thiocyanate from Panreac (Barcelona, Spain). Gallic acid, 2,2′-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid diammonium salt (ABTS), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2–carboxylic acid (Trolox), NBT (nitrobluetetrazolium), Bovine Serum Albumin (BSA), Xanthine and Xanthine-oxidase from Sigma-Aldrich (Gillingham, UK).

Extraction of *Gentiana Lutea*

Dried roots of *Gentiana Lutea* were finely ground using a standard kitchen food processor. Ground *Gentiana Lutea* (5 g) was extracted in two ways; (1) with 50:50 (v/v) methanol:water and (2) with water, always in the ratio 1:10 (w/v). The extraction was performed at 4 ± 1 °C for 24 h, in the dark with constant stirring. The extract solutions of *Gentiana Lutea* were recovered by filtration using Whatman Filter paper, 0.45 μm. Part of the supernatant was taken for subsequent use to determine the antiradical capacity. The

volume of the remaining supernatant was measured and the excess methanol was removed under vacuum using a rotary evaporator (BUCHI RE111, Postfachi, Switzerland) and kept frozen at −80 °C for 24 h. All extracts were dried in a freeze dryer (Unicryo MC2L −60 °C, Martinsried, Germany) under vacuum conditions at −60 °C for 3 days to remove moisture. Finally, *Gentiana Lutea* lyophilize (freeze dried) were weighed to determine the concentration recovered (g/L) and the extraction yield (%) as Zhang et al. (2007)[18]. Samples were then weighed and kept protected from light in a desiccator until use.

Determination of the Total Phenolic Content (TPC)

The Folin-Ciocalteu method was used to determine the total phenolic content (TPC) as reported by Santas et al. (2008)[19]. The sample was diluted 1:25 (v/v) in order to be in the range of absorbance. The final concentration (v/v) for the mixture was; sample 7.7%, Folin reagent 4% and saturated sodium carbonate solution 30.8%. The mixture was finally diluted with Mili Q water, shaken and incubated in the dark for 1 h. Absorbance at 765 nm was measured using a microplate reader (Fluostar Omega, BMG Labtech, Ortenberg, Germany) against water as a blank. Gallic acid was used to prepare a standard calibration, and the results were expressed as mg of Gallic acid equivalents/g dry weight (mg GAE/g DW).

Determination of Free Radical Scavenging Activity Assays

a. TEAC Assay

The antioxidant capacities of *Gentiana Lutea* were measured by using a modified TEAC assay, which was performed as described by Miller et al. (1996)[20]. The TEAC assay was based on the reduction of the $ABTS^{\bullet+}$ radical cation by the antioxidants present in the samples. $ABTS^{\bullet+}$ radical cation (7 mM, final concentration) was dissolved before adding potassium sulphate (2.45 mM, final concentration) and allowing the mixture to stand in the dark up to 16 h. Phosphate Buffer Solution (PBS, 10 mM) with the $ABTS^{\bullet+}$ radical cation was incubated at room temperature for 30 min before used. Then, the mixture of the $ABTS^{\bullet+}$ radical cation was adjusted to an absorbance of 0.73 ± 0.2 nm, using a microplate reader (Fluostar Omega, BMG Labtech, Ortenberg, Germany). The TEAC values for the different concentrations of each compound were interpolated from the trolox calibration curve and

expressed as milligrams of trolox equivalent per gram of dry weight sample (mg TE/g DW sample).

b. DPPH Assay

The effect of the extracts on the scavenging of DPPH radical was determined according to the method adapted from Madhujith et al. (2006) with slight modifications[21]. The sample was diluted 1:20 (v/v) and DPPH radical in methanol (5.07 mM) was made for the study. Then, the sample (10% v/v) and DPPH solution (90% v/v) were added to the well of the microplate. The absorbance was measured at 517 nm over every 15 min for 75 min. The results were expressed as mg TE/g DW sample.

c. Superoxide Activity Xanthine/Xanthine Oxidase (X/XO)

The method was based on the developed method of Valentao et al. (2001)[22] and modified for application in microplates by Lopez et al. (2001)[23]. All test samples were dissolved in a 50 mM phosphate buffer to simulate the environment in which the reaction occurs in the body. The sample was mixed with 145 µM of a solution of xanthine, 50 µM of a solution of NBT and incubated in 37 °C. The sample extract was diluted from 1:10 to 1:100 (v/v) for the study. Finally, 0.29 U/mL of enzyme xanthine oxidase solution was added and the absorbance was recorded at 560 nm every 2 min. The value of IC_{50} was calculated to determine the inhibition rate of *Gentiana Lutea* in the reaction.

Determination of Antioxidant Activity in o/w Emulsion

a. Removal of Tocopherols from Sunflower Oil

Alumina was placed in an oven at 200 °C for 24 h, and then removed and allowed to cool in a desiccator until it reached room temperature. Sunflower oil triacylglycerol was passed twice through the alumina in a column to remove the tocopherols as described by Yoshida et al. (1993)[24]. Finally, the filtered oil was stored at −80 °C until use.

b. Preparation of Emulsion

Oil in water emulsion was prepared by dissolving Tween-20 (1%, final concentration) in Milli Q water and adding oil (10%, final concentration). To form an emulsion, the oil was added drop wise to the solution of Tween-20 and water, which was kept cold, and sonication

process was continued for 5 min. All samples were redissolved in ethanol-50% (v/v) to obtain the final concentration in the emulsion. The final samples were prepared either (i) control (no addition); (ii) 0.35% (w/w) Trolox (positive control); (iii) 0.1% (w/w) BSA; (iv) 0.5% (w/w) lyophilise *Gentiana Lutea* ; (v) 0.5% (w/w) lyophilise *Gentiana Lutea* mixed with 0.1% (w/w) BSA; (vi) 0.2% (w/w) lyophilise *Gentiana Lutea* and (vii) 0.2% (w/w) lyophilise *Gentiana Lutea* mixed with 0.1% (w/w) BSA. The emulsion for each sample was prepared in quadruplicate, obtaining a total of 28 samples and stored in the dark and allowed to oxidize at 37 °C. The pH of the samples was measured four times for each sample (pH meter GLP21, Crison Instruments, Barcelona, Spain) as a parameter to investigate its correlation with PV.

c. Determination of Peroxide Value (PV)

The primary oxidation products were measured using peroxide value (PV) according to the thiocyanate method of the Association of Official Analytical Chemists (AOAC) 8195[25]. Ferrous chloride solution was prepared in hydrochloric acid (1 M) with the addition of iron chloride (II) (2 mM, final concentration). Ammonium thiocyanate solution was prepared in water (2 mM, final concentration). The assay was performed with a drop of emulsion in the range from 0.007 to 0.01 g, diluted with ethanol. From this solution the required amount of sample, varying according to the degree of oxidation, was taken in a cuvette and ethanol (96%) was added. Ferrous chloride and ammonium thiocyanate solutions were added, each in a proportion of 1.875% (v/v), final concentration. The absorbance was measured spectrophotometrically at λ = 500 nm. The results are expressed as meq hydroperoxides/kg of emulsion.

d. Determination of Secondary Oxidation by Thiobarbituric Acid Reactive Substances TBARS

The TBARS method was adapted from Gallego et al. (2013)[26]. The TBARS reagent was prepared (15% w/v trichloroacetic acid, 0.375% w/v thiobarbituric acid and hydrochloric acid 2.1% v/v). One mL of each emulsion was taken and the TBARS reagent was added in the ratio 1:5 (v/v). Immediately the samples were added to an ultrasonic bath (5 min) and after immersing in a water bath preheated to 95 °C (20 min) the samples were centrifuged and the absorbance of the supernatant was measured at λ= 531 nm. The results are expressed as mg malondialdehyde (MDA)/kg of emulsion.

Statistical Analysis

Statistical analysis was performed using a one-way analysis of variance ANOVA using Minitab 16 software program (Minitab, Inc., Paris, France). When a statistically significant difference was found, Tukey's tests were performed and the statistical significance was set at $p < 0.05$. The results were presented as mean values ($n \geq 3$).

HPLC and Post-Column HPLC-ABTS$^{\bullet+}$ Radical Scavenging Method

The method for identification of peaks with antioxidant activity was that used by Koleva et al. (2001) with some modifications[27]. The instrument was a Waters 2695 separations module (Meadows Instrumentation, Inc., Bristol, USA) system with a photodiode array detector Waters 996 (Meadows Instrumentation, Inc., Bristol, USA). The column used was a Kinetex C18 100A, (100 × 4.6 mm, Phenomenex, Torrence, CA, USA). Solvents used for separation were 0.1% acetic acid in water (v/v) (eluent A) and 0.1% acetic acid in methanol (v/v) (eluent B). The gradient used was isocratic, 75% A. The flow rate was 0.6 mL/min. Detection wavelength was 230 nm (to see the peaks) and 734 nm (to see the ABTS radical). The sample injection volume was 10 µL. The chromatographic peaks of gentiopicroside and sweroside were confirmed by comparing their retention times and diode array spectra with that of their reference standards. The pump for ABTS post-column injection was a Merk-Hitachi HPLC gradient pump (Model L-6200, Hitachi High Technologies America, Inc., Schaumburg, Illinois, USA) with a 0.2 mL/min flow; ABTS concentration was of 0.03% (w/v).

3.3.3 Results and Discussion

Analysis of Total Polyphenols and Free Radical Activity Assays

On average, from 5 g of dried *Gentiana Lutea* extracted with aqueous methanol 50:50 (v/v) and water alone, it was possible to recover 1.5 ± 0.05 g and 1.0 ± 0.04g of lyophilised, respectively. The concentration recovered was proportional to the extraction yield shown in Table 1. Previous study reported that gentiopicroside compound, an active compound that

signifies the main bitter principle in *Gentiana Lutea* was still preserved at almost 83.5% after drying[28].

Table 1. Extraction yield, total phenolic content (TPC), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Trolox equivalent capacity assay (TEAC) and enzymatic activity of *Gentiana Lutea*

Activity *Gentiana Lutea*	**Extraction solvent**	
	H_2O	**50:50 MeOH:H_20**
Extraction yield (%)	20.00 ± 0.9%	29.10 ± 0.3%
Total phenolic content (g GAE / g DW)	3.79 ± 1.7	12.03 ± 1.8
DPPH (µmol of TE / g DW)	12.34 ± 1.5	15.89 ± 0.5
TEAC (µmol of TE / g DW)	33.28 ± 1.5	48.90 ± 1.8
Superoxide activity (mg / ml)	30.00 ± 2.8	23.21 ± 2.8

Mean value n = 3 and the standard deviation for each assay is less than 5%. Gallic Acid Equivalent (GAE), Trolox Equivalent (TE), Dry Weight (DW).

The concentration of total polyphenols and the value of antioxidant activity assays were determined and the results are shown in Table 1. The extract of *Gentiana Lutea* in methanol-50% showed higher phenolic content and antioxidant activity than the water extract. The total phenolic content of *Gentiana Lutea* extracts allowed the estimation of all phenolic acids, flavonoids, anthocyanins, nonflavonoids and many classes of polyphenol compounds present in the samples. On the other hand, Nastasijevic et al. (2012) determined the total polyphenol content of *Gentiana Lutea* in water extract as being slightly higher compared to different concentrations of aqueous ethanol and methanol extracts[10].

Water extracts of *Gentiana Lutea* showed the lowest activity in free $DPPH^{\bullet}$ scavenging activity compared to methanol-50% extract, similar to previous research from

Kintzios et al. (2010)[14]. It is not the first time that the antioxidant activity of *Gentiana Lutea* by DPPH method has been carried out. However, variation in results may be due to the plant age, solvent, method and system used throughout the experiment[10,14,29].

For the TEAC assay, the finding was consistent with the DPPH method where the aqueous methanol extract showed higher activity than the water extract. TEAC assay indicated the extract potency used as a source of antioxidants based on the ability of the antioxidant compound to scavenge the long-life radical cation $ABTS^{\bullet+}$. In the results shown in Table 1 it can be appreciated that the methanol-50% extract has a higher capacity to scavenge $ABTS^{\bullet+}$ radicals and consequently shows a higher antioxidant activity than DPPH assay. To the best of our knowledge, this is the first report of the antioxidant activity of extracts from *Gentiana Lutea* roots assessed using the TEAC methods.

Some of the previous reports showed that the antioxidant activity of plant extracts correlates with the phenolic content[30] and the yield of phenolic components from herbs is higher with methanol-50% rather than with water as extract. The mixtures of alcohol and water have been more efficient in extracting compounds and give a better yield than the corresponding mono-component solvent system. Xanthones such as isogentisin and gentisin and its derivatives are one of main sources of phenolic compounds in *Gentiana Lutea* and are expected to be more soluble in aqueous alcohol. A study showed good correlation between phenolic content and antioxidant activity[31] whereas another found no correlation[32].

In the present work, an effective antioxidant activity in *Gentiana Lutea* was found. Methanol-50% extract exhibited $O_2^{\bullet-}$ scavenging activity, measured using the X/XO system (Table 1), with an IC_{50} at 23.21 ± 2.8 mg/mL. Water extract of *Gentiana Lutea* showed lower scavenging activity than methanol aqueous extract, with IC_{50} = 30.00 ± 2.8 mg/mL. These results are consistent with Kusar et al. (2006)[29], who demonstrated the effect of superoxide activity of *Gentiana Lutea* leaf and root in methanol extracts, with IC_{50} inhibition value of 11.1 mg/mL and 8.2 mg/mL, respectively. Kusar and co-workers' findings were accomplished by X/XO reaction mixture with DEPMO-OOH scavenger that transformed the reaction to a stable radical measured by electro spin resonance (ESR). Valentao et al. (2001)[22] observed the phenolic acids (*p*-coumaric acid, ferulic acid, sinapic acid and kaempferol) exhibited superoxide scavenger activity and an inhibitory effect on XO. Considering the results obtained from TPC assay, it may be anticipated that *Gentiana Lutea* extract has antioxidant activity achieved by the scavenging of superoxide radical and XO inhibition.

Antioxidant Effect in Stored o/w Emulsion

Many strategies on laboratory scale have been developed to improve the stability of shelf life in food models including adding a minimum amount of natural plants to delay the oxidation rate. The effect of *Gentiana Lutea* on inhibiting lipid oxidation in oil-in-water (o/w) emulsion as a food model has not been described. In this study it also has been carried out to determine the synergic effect of *Gentiana Lutea* with BSA in o/w emulsions. The oxidation in o/w emulsions was measured in two stages of oxidation; primary oxidation product (Peroxide Value) and secondary oxidation products (TBARS). In addition the change in pH was monitored, since pH tends to fall during oxidation.

a. Evolution of Peroxide Value (PV)

Figure 1 (below) shows the evolution of PV *vs*. time. The control (without extract added) showed the highest oxidation throughout the storage time followed by the emulsion with only BSA (0.1%). The sample containing Trolox (0.35%, positive control) and the samples containing extracts, were not oxidized during the first 10 days. They show significant difference from the control ($p < 0.05$). The time required for the emulsions to reach a peroxide value of 10 meq hydroperoxides/kg of emulsion was determined as a standard to measure the stability of emulsion. The limits of fat product (animal, plant and anhydrous) margarine and fat preparation were set <10 meq hydroperoxides/kg as a guarantee of the product quality[33]. When the peroxide value of the sample is measured as greater than 15 meq hydroperoxide/kg, the sample is considered rancid, which may alter the color, taste and nutritional quality due to the deterioration of the lipid.

The control was the first sample to reach 10 meq hydroperoxides/kg of emulsion which occurred rapidly in two days. The emulsion with BSA exhibited a similar deterioration rate to the control, revealing that BSA, in this concentration of 0.1%, does not provide any antioxidant effect in the emulsions. Positive control samples (Trolox) showed good antioxidant effect over 11 days and begin to oxidize rapidly after 15 days, reaching 88 meq hydroperoxides/kg emulsion on the final days of the experiment.

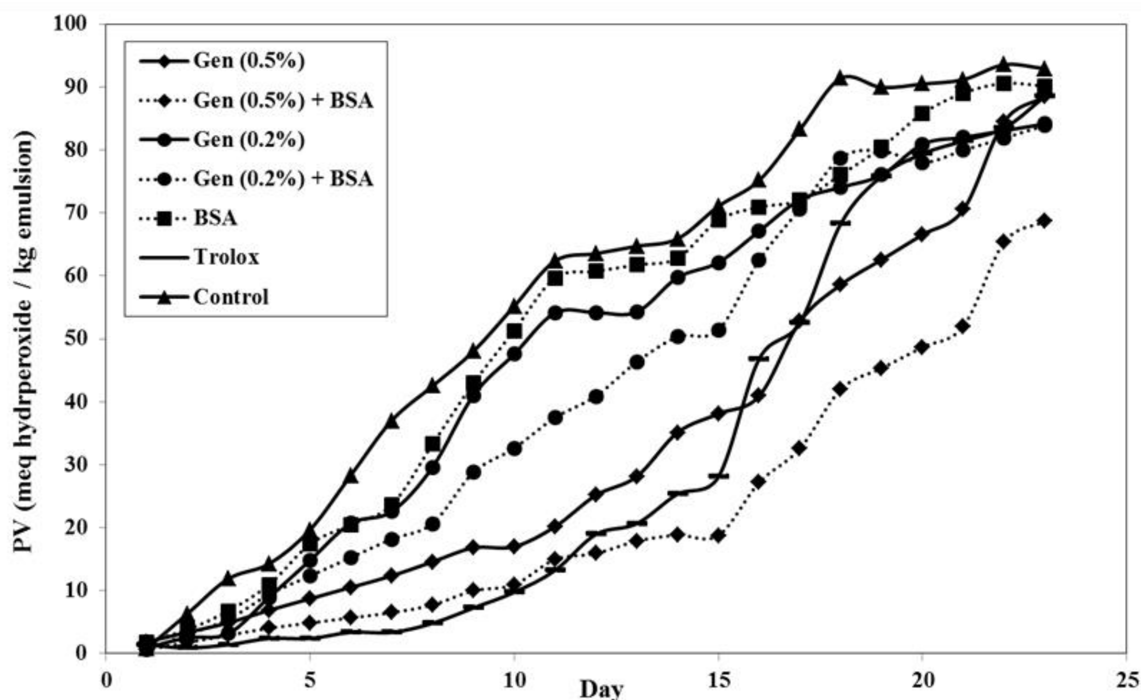


Figure 1. Change of peroxide value over time stored at 37 °C (each value is expressed as mean ($n = 3$)).

Adding 0.2% *Gentiana Lutea* to the emulsion, with or without adding BSA, did not result in any relevant effect towards oxidation in the first stage (PV <10 meq hydroperoxides/kgin time <3 days). There is a significant difference between 0.2% antioxidant sample with BSA and in its absence ($p < 0.05$). The sample with 0.5% *Gentiana Lutea* showed antioxidant activity towards lipid degradation first at 15 days and gradually oxidized after 15 days ($p < 0.05$). Finally, the sample with *Gentiana Lutea* 0.5% and BSA 0.1% displayed the lowest PV, with significant differences with the other samples throughout storage time ($p < 0.05$); it took almost 10 days to reach above 10 meq hydroperoxides/kg of emulsion.

Almajano et al. (2004) reported that some antioxidant compounds such as EGCG and caffeic acid mixed with BSA cause a marked increase of the antioxidant activity in an emulsion[34]. Since BSA is known to be surface active, the increase of antioxidant activity in emulsions containing a mixture of antioxidant and BSA could be due to BSA binding with the antioxidant and transporting it to the oil water interface, where it is highly effective in reducing the rate of oxidation[35]. The authors also stated that the antioxidant molecule had bound to the BSA protein, proved by TEAC assay, and showed a progressive increase in the

radical scavenging ABTS$^{\bullet+}$ with the storage time over several days[34]. The mixture of 0.1% BSA and 0.5% *Gentiana Lutea* in emulsion exhibit the lowest oxidation rate compared to all samples shown in this experiment. These results showed for the first time the important effect of *Gentiana Lutea* extract on lipid oxidation with synergic effect to BSA tested in an o/w emulsion. This concentration of 0.5%, demonstrated the best antioxidant effect throughout the storage period.

b. Evolution of pH over Time

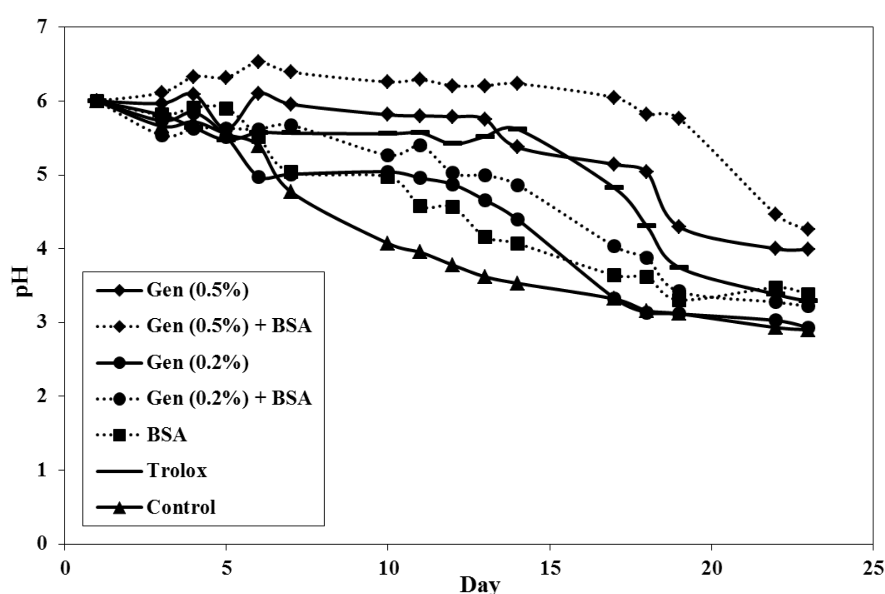


Figure 2. Change of pH over time, stored at 37 °C (each value is expressed as mean (n = 3)).

Since decomposition of hydroperoxide measured in PV assay is acidic, the pH change in the sample is considered inversely proportional to the PV. Thus, the pH measured is a parameter of which its correlation with PV can be investigated. Antioxidant activity in food models is less effective under low pH conditions. However, some antioxidant compounds such as carnosic acid and carnosol (found in rosemary) have been reported to have high antioxidant activity at lower pH, which is at pH 4–5[36]. Figure 2 shows the changes of pH value on emulsions over 23 days storage. Overall, the decrease in pH value throughout storage was of a similar order with increased primary oxidation measured in the PV assay. These results agreed with the studies done by Frankel et al. (1997)[16]. They found that the lipid oxidation in emulsion is slower at higher pH, and decreased when oxidation is

accelerated. All samples started nearly at neutral pH and 0.5% *Gentiana Lutea* with 0.1% BSA showed the highest value throughout storage. Similar to PV, the pH of the sample with 0.2% *Gentiana Lutea* with or without BSA showed higher pH than control but the value was not significant throughout storage ($p > 0.05$). The pH of 0.5% *Gentiana Lutea* with BSA and 0.5% *Gentiana Lutea* alone, showed significant differences compared to all samples during storage period ($p < 0.05$). The behavior of pH of the sample with 0.5% *Gentiana Lutea* with BSA was stable until 19 days before it started to decrease.

Skowyra et al. (2013) demonstrated that the pH and PV have the best correlation with $R^2 = 0.9648$[37]. Our results are in agreement with them and it can be described that the antioxidant activity in o/w emulsion which is stable at pH 6 showed an inverse relationship at a lower PV value. Some authors also reported a similar agreement of pH change which was inversely proportional to the lipid oxidation[26,38,39]. Meanwhile, Mancuso *et al*. (1999) suggested the initial oxidation of emulsion depended on pH, by varying the effect of emulsifier[40, 41]. The authors observed a higher oxidation rate occurring at pH 7 rather than pH 3 o/w emulsion. Results may be due to the iron solubility increasing at low pH and allowing iron to be partitioned into the continuous phase, whereas insoluble iron at high pH may precipitate onto the emulsion droplet surface resulting in an increase in the lipid oxidation.

c. Evolution of Thiobarbituric Acid Reactive Substances (TBARS)

One of the compounds produced from secondary oxidation in lipids is MDA (malondialdehyde) which can be measured by the TBARS method. The secondary lipid oxidation is responsible for the alteration of flavor, rancid odor and the undesirable taste in foods[42].

Secondary oxidation products were monitored by TBARS assay and are shown in Figure 3. Similar to PV, the control had the most rapid increase in TBARS followed by the BSA sample. TBARS values for samples treated with 0.5% gentian powder, with and without BSA, experienced below 1.2 mg MDA /kg sample over the first 21 days and showed prominently lower than positive control up to 4 weeks ($p < 0.05$). The sample with 0.2% of *Gentiana Lutea* alone does not display significant delay in lipid oxidation ($p > 0.05$), meanwhile 0.2% *Gentiana Lutea* with BSA and positive control showed significant different during 20 days ($p \leq 0.05$). From the TBARS results exhibited, the synergic effect between both the concentration of *Gentiana Lutea* and BSA in the emulsion during the storage time is

demonstrated and the samples with both (gentian and BSA) show the lowest oxidation rate compared to all samples.

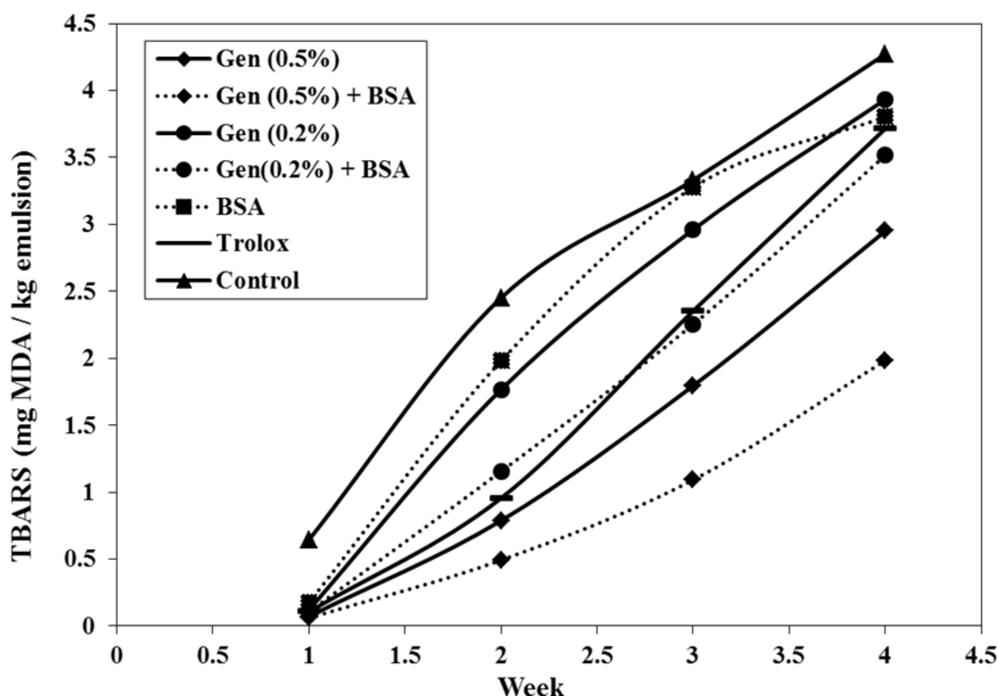


Figure 3. Change of Thiobarbituric Acid Reactive Substances (TBARS) over time, stored at 37 °C (each value is expressed as mean (n = 3)).

After 23 days, all samples are oxidized with above 1.2 mg malondialdehyde/kg sample even though *Gentiana Lutea* with the BSA mixture showed minimum rise and had the best antioxidant effect in the emulsion. This behavior is not new. It has been previously reported that artificial antioxidants such as Trolox, epigallocatechingallate (EGCG), caffeic acid are more stable in emulsions during storage in the presence of BSA than in its absence[34].

Gentiana Lutea has compounds which of family of iridoids, scoiridoids and xanthones[3]. It could be developed as antioxidant agent and radical scavengers and may contribute to the decrease of lipid oxidation[43]. The water soluble antioxidant molecular structures differ in the number of phenolic hydroxyl groups, their location and the carboxylic acid group[34]. Thus, the range of structure presented may allow any possible interaction with the BSA to be detected.

Many findings determined such amount of natural plants work as antioxidant efficiently; depended on variation of system tested and plant preparation. However, the more important is to identify the minimum concentration required to reduce lipid oxidation significantly throughout storage time. Adding minimum amount of natural plant not only can delay lipid oxidation, but also to avoid some changes on sensorial quality and flavored. Analysis on our data showed for *Gentiana Lutea* required as minimum 0.5% (w/w) would be beneficial for reducing the velocity of lipid oxidation significantly in both primary and secondary oxidation in emulsion system ($p < 0.05$). Adding also 0.1% (w/w) of BSA gave better effect of the antioxidant activity towards emulsion, compare to sample with *Gentiana Lutea* alone. The study confirmed the potential of edible *Gentiana Lutea* to prevent oxidation of emulsion.

HPLC Analysis of Gentiana Lutea and the Total Antioxidant Activity Based on Post-Column On-Line Coupling ABTS$^{\bullet+}$

The HPLC analysis is targeted to identifysecoiridoid-glycoside, the bitter constituent occurred in the extract shown in (a) and (b). Results in Table 2 present secoiridoid glycoside; gentiopicroside, swerosideand amarogentin were the important compounds in the *Gentiana Lutea*. The highest content of secoiridoidin *Gentiana Lutea* extract in methanol-50% weregentiopicroside (1805 ± 62 mg/L extract) and the amount of sweroside found was 72 ± 4 mg/L extract. It was not possible to identifyamarogentin in the extract, meanwhile only low traces of amarogentinwere found in various commercial *Gentiana Lutea* (less than 0.09%)[3]. Carnat et al. (2005) also reported that amarogentin can only be found in some fresh root of *Gentiana Lutea*[28]. There are comprehensive studies measuring the active compound in *Gentiana Lutea* using bioassay-guided fractionation such as HPLC[3], Capilary Electrophoris[44] and Thin Layer Chromatography[45]. From the literature study, there is constituent of secoiridoid that has not been identified in this study (swertiamarin) are believed to be existed in the extract[2]. This is most likely due to the objective of the method was not optimize the quantification of the traces but to measure the antioxidant activity of each compound in the *Gentiana Lutea* extract.

Table 2. Amount of secoiridoid-glycoside quantified by HPLC.

Sample	Concentration (mg / L)
a) Gentiopocroside	1805 ± 62
b) Sweroside	72 ± 4
c) Amarogentin	n.d

n.d = not detected.

Aberham et al. (2007) analyzed the active compound of 12 commercial samples of *Gentiana Lutea* root[3]. They found that swertiamarin was shown to have consistent occurrence between 0.21% and 0.45% and gentiopicroside was the most dominant compound in the sample up to 9.53%. Meanwhile, Ando et al. (2007) reported that gentiopicroside was not detected from the fresh roots of 3-year-old *Gentiana Lutea*[46]. In contrast, Hayashi et al. (1990) described that one year root contains high amounts of gentiopicroside and amarogentin and decreases over 5 years[47]. The Gentianaceae family is well known for its intensive bitter root used as a tonic for the digestive system with many pharmacological benefits. There are other plants such as *Swertiachirayita*[48] and *Lonicera japonica*[49] that also possess similar compounds of secoiridoid-glycosides (swertiamarin and sweroside).

Investigation of the main individual compounds in *Gentiana Lutea* root was previously developed and optimized by Aberham et al. (20011,2007)[2,3]. Furthermore, a substantial number of studies have demonstrated the effect of the secoiridoid group on the scavenging function to generate free radicals[7,13,48]. Wei et al. (2012) reported that five secoiridoids, including gentiopicroside, sweroside, swertiamarin and sweroside, did not show any scavenging ability towards free radicals by *in vitro* DPPH assay. However, taking into account their report, it was desirable to explore more individual extracted compounds by isolating the compounds followed by a biochemical assay, such as ABTS radical, to measure their activity. Online post-column methods are very dependable because they combine systems for investigating different features of the sample simultaneously. Our initial observation of using *in vitro* ABTS assay showed that gentiopicroside and sweroside displayed no scavenging activity towards ABTS radicals (data not shown) while the activity of these compounds is similar towards DPPH radicals. However, our finding showed an activity of amarogentin analyzed by ABTS *in-vitro* assay (644.5 ± 17.5 mg eq TE/L sample).

However, the amarogentin was not identified in the extract, as discussed above. This is in contrast with Phoboo et al. (2013) who observed no scavenging activity of amarogentin towards DPPH radical[48].

The HPLC separated analytes reacted with ABTS radical post column (see Figure 4) and the reduction was detected as a negative peak at 734 nm. In Figure 5, the chromatographic analysis showed gentiopicroside (a) and sweroside (b) detected in *Gentiana Lutea* extract and unknown compounds, (c) and (d), detected as negative peaks using the ABTS radical assay, which indicated that these components had free radical scavenging activity. The result of antioxidant response peaks (negative peak) of the *Gentiana Lutea* compounds, expressed as mg galic acid equivalent (GAE)/L extract, indicates their relative contribution to the antioxidant activity of the extract with concentration taken into account. Analysis of *Gentiana Lutea* extract of total antioxidant activity had been reported several times mainly measured by DPPH *in vitro* assay[10, 14]. Even though we were unable to identify the compound relevant to the scavenging activity in the post-column ABTS assay, the results showed that the antiradical capacity of *Gentiana Lutea* is not related to gentiopicroside and sweroside. There are many other compounds that maybe related to the scavenging activity in the extract such as xanthone-glycosides.

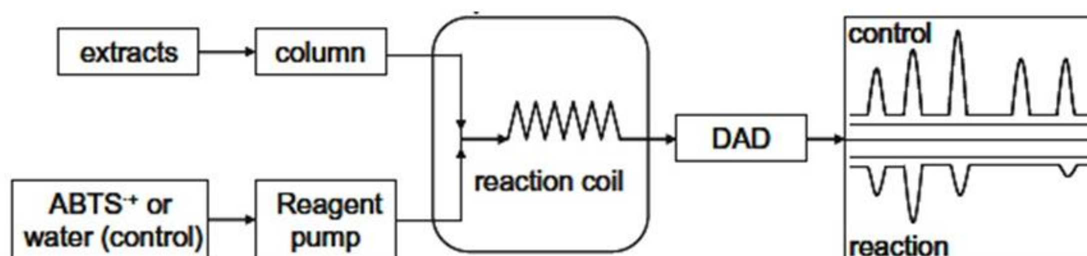


Figure 4. Scheme of HPLC-ABTS for screening of antioxidant compounds in *Gentiana Lutea* root extract.

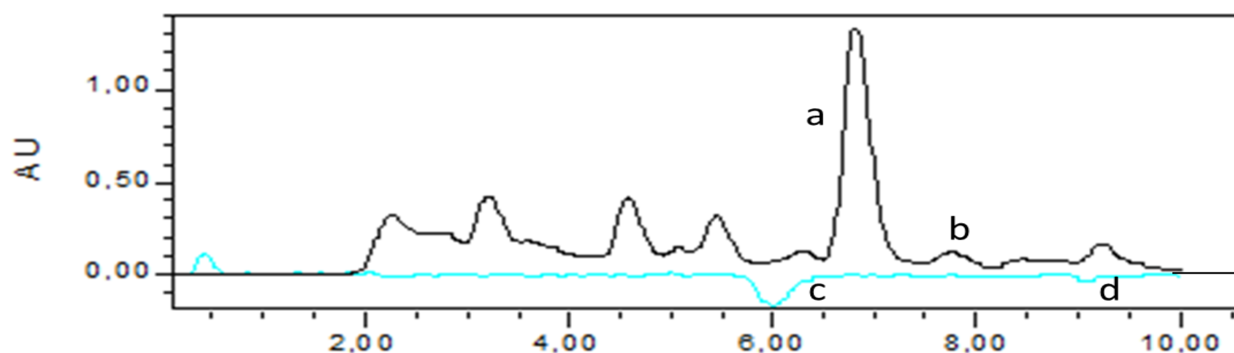


Figure 5. Chromatogram of *Gentiana Lutea* root extract obtained direct and from the post-column HPLC-ABTS$^{•+}$ radical scavenging method. (a) Gentiopicroside; (b) Sweroside; (c) antiradical activity by unknown compound; 31.33 ± 1.16 mg GAE/L and (d) antiradical activity by unknown compound; 8.30 ± 0.12 mg GAE/L.

Nevertheless, our finding showed that the 0.5% *Gentiana Lutea* extracts is able to delay the process of oxidation and give better storage stability as emulsion. However, this activity is not due to the bitter compounds (gentiopicroside amarogentin and sweroside) presented in our extract. Studies (from the revised literature) proved that xanthone compounds such as gentioside, gentisin and isogentisin found in *Gentiana Lutea* have antiradical activity even though their constituents possess remarkable activity in pharmacological study.

3.3.4 Conclusions

Gentiana Lutea has valuable pharmacological properties due to the bitter properties of its secoiridoid-glycosides; their extraction using methanol-water mixture was better than water alone. *Gentiana Lutea* extract showed excellent antioxidant activity in aqueous methanol measured by DPPH scavenging activity assay and Trolox equivalent capacity assay (TEAC) methods (15.89 and 48.90 µmol of TE/g DW, respectively). *Gentiana Lutea* lyophilize can be applied as antioxidants in oil-in-water emulsions. 0.2% (w/w) of lyophilize. *Gentiana Lutea* does not inhibit lipid oxidation significantly. An amount of 0.5% (w/w) *Gentiana Lutea* lyophilises exhibited antioxidant activity towards primary and secondary oxidation in an o/w emulsion. Adding 0.1% (w/w) BSA with *Gentiana Lutea* in an emulsion showed a synergic effect and better activity in delaying lipid oxidation. Gentiopicroside and

sweroside found in HPLC analysis do not show any antiradical capacity in *Gentiana Lutea* aqueous methanol extract. However, total antiradical capacities shown in post-column measurements presented activities of 31.33 ± 1.16 mg GAE/L and 8.30 ± 0.12 mg GAE/L towards the ABTS free radical. This study confirmed that *Gentiana Lutea* roots as a source of edible natural antioxidants have potential to be used by the food industry.

References

1. Balijagić J, Janković T, Zdunić G, Bosković J, Savikin K, Godevac D, Stanojković T, Jovancević M, Menković N. Chemical profile, radical scavenging and cytotoxic activity of yellow gentian leaves (*Genitaneae luteaefolium*) grown in northern regions of Montenegro. *Nat. Prod. Commun.* 2012;7:1487–1490.

2. Aberham A, Pieri V, Croom EM, Ellmerer E, Stuppner H. Analysis of iridoids, secoiridoids and xanthones in *Centaurium erythraea*, *Frasera caroliniensis* and *Gentiana Lutea* using LC-MS and RP-HPLC. *J. Pharm. Biomed. Anal.* 2011;54:517–525.

3. Aberham A, Schwaiger S, Stuppner H, Ganzera M. Quantitative analysis of iridoids, secoiridoids, xanthones and xanthone glycosides in *Gentiana Lutea* L. roots by RP-HPLC and LC-MS. *J. Pharm. Biomed. Anal.* 2007;45:437–442.

4. Lian LH, Wu YL, Wan Y, Li X, Xie WX, Nan JX. Anti-apoptotic activity of gentiopicroside in D-galactosamine/Lipopolysaccharide-induced murine fulminant hepatic failure. *Chem. Biol. Interact.* 2010;188:127–133.

5. Pal D, Sur S, Mandal S, Das A, Roy A, Das, S, Panda CK. Prevention of liver carcinogenesis by amarogentin through modulation of G 1/S cell cycle check point and induction of apoptosis. *Carcinogenesis.* 2012;33:2424–2431.

6. Tan RX, Kong LD, Wei HX. Secoiridoid glycosides and an antifungal derivative from gentian 4 tibetica. *Phytochemistry.* 1998;47:1223–1226.

7. Kumarasamy Y, Nahar L, Sarker S. Bioactivity of gentiopicroside from the aerial parts of Centaurium erythraea. *Fitoterapia.* 2003;74:151–154.

8. Jia N, Li Y, Wu Y, Xi M, Hur G, Zhang X., Cui J, Sun W, Wen A. Comparison of the anti-inflammatory and analgesic effects of *Gentiana macrophylla* Pall. and *Gentiana straminea* Maxim., and identification of their active constituents. *J. Ethnopharmacol.* 2012;144:638–645.

9. Mihailović V, Mihailović M, Uskoković A, Arambašić J, Mišić D, Stanković V, Katanić J, Mladenović M, Solujić S. Matić S. Hepatoprotective effects of *Gentiana asclepiadea* L. extracts against carbon tetrachloride induced liver injury in rats. *Food Chem. Toxicol.* 2013;52:83–90.

10. Nastasijević B, Lazarević-Pašti T, Dimitrijević-Branković S, Pašti I. Vujačić A, Joksić G, Vasić V. Inhibition of myeloperoxidase and antioxidative activity of *Gentiana Lutea* extracts. *J. Pharm. Biomed. Anal.* 2012;66;191–196.

11. Jiang DJ, Dai Z, Li YJ. Pharmacological effects of xanthones as cardiovascular protective agents. *Cardiovasc. Drug Rev.* 2004;22:91–102.

12. Rana VS, Rawat MSM. A new xanthone glycoside and antioxidant constituents from the rhizomes of *Swertia speciosa*. *Chem. Biodivers.* 2005;2:1310–1315.

13. Wei S, Chen G, He W, Chi H, Abe H, Yamashita K, Yokoyama M, Kodama H. Inhibitory effects of secoiridoids from the roots of *Gentiana straminea* on stimulus-induced superoxide generation, phosphorylation and translocation of cytosolic compounds to plasma membrane in human neutrophils. *Phytother. Res.* 2012;26:168–173.

14. Kintzios S, Papageorgiou K, Yiakoumettis I, Baricevic D, Kusar A. Evaluation of the antioxidants activities of four Slovene medicinal plant species by traditional and novel biosensory assays. *J. Pharm. Biomed. Anal.* 2010;53:773–776.

15. Estévez M, Ventanas S, Cava R. Food chemistry and toxicology protein oxidation in frankfurters with increasing levels of added rosemary essential oil: Effect on color and texture deterioration. *J. Food. Sci.* 2005;70:427–432.

16. Frankel EN, Huang S, Aeschbach R. Antioxidant activity of green teas in different lipid systems. *J. Am. Oil Chem. Soc.* 1997;74:1309–1315.

17. Kiokias S, Dimakou C, Oreopoulou V. Activity of natural carotenoid preparations against the autoxidative deterioration of sunflower oil-in-water emulsions. *Food Chem.* 2009;114;1278–1284.

18. Zhang S, Bi H, Liu C. Extraction of bio-active components from *Rhodiola sachalinensis* under ultrahigh hydrostatic pressure. *Sep. Purif. Technol.* 2007;57:277–282.

19. Santas J, Carbo R., Gordon M, Almajano M. Comparison of the antioxidant activity of two Spanish onion varieties. *Food Chem.* 2008;107:1210–1216.

20. Miller NJ, Sampson J, Candeias LP, Bramley PM, Rice-Evans CA. Antioxidant activities of carotenes and xanthophylls. *FEBS Lett.* 1996;384:240–242.

21. Madhujith T, Shahidi F. Optimization of the extraction of antioxidative constituents of six barley cultivars and their antioxidant properties. *J. Agric. Food Chem.* 2006;54:8048–8057.

22. Valentão P, Fernandes E, Carvalho F, Andrade PB, Seabra RM, Bastos ML. Antioxidant activity of *Centaurium erythraea* infusion evidenced by its superoxide radical scavenging and xanthine oxidase inhibitory activity. *J. Agric. Food Chem.* 2001;49:3476–3479.

23. López-Cruz RI, Zenteno-Savín T, Galván-Magaña F. Superoxide production, oxidative damage and enzymatic antioxidant defenses in shark skeletal muscle. *Comp. Biochem. Physiol.* 2010;156:50–56.

24. Yoshida H, Kajimoto G, Emura S. Antioxidant effects of D-tocopherols at different concentrations in oils during microwave heating. *J. Am. Oil Chem. Soc.* 1993;70:989–995.

25. American Oil Chemists' Society. *AOCS Official Method Cd 8-53*; Firestone, D., Ed.; Official Methods and Recommended Practices of the American Oil Chemists' Society: Champaign, IL, USA, 1997.

26. Gallego MG, Gordon MH, Segovia FJ, Skowyra M, Almajano MP. Antioxidant properties of three aromatic herbs (rosemary, thyme and lavender) in oil-in-water emulsions. *J. Am. Oil Chem. Soc.* 2013;90:1559–1568.

27. Koleva II, Niederländer HA, van Beek T. Application of ABTS radical cation for selective on-line detection of radical scavengers in HPLC eluates. *Anal. Chem.* 2001;73:3373–3381.

28. Carnat A.Fraisse D, Carnat AP, Felgines C, Chaud D, Lamaison JL. Influence of drying mode on iridoid bitter constituent levels in gentian root. *J. Sci. Food Agric.* 2005;85:598–602.

29. Kusar A, Zupancic A, Sentjurc M, Baricevic D. Free radical scavenging activities of yellow gentian (*Gentiana Lutea* L.) measured by electron spin resonance. *Hum. Exp. Toxicol.* 2006;25:599–604.

30. Almajano MP, Carbó R, Jiménez JAL, Gordon MH. Antioxidant and antimicrobial activities of tea infusions. *Food Chem.* 2008;108:55–63.

31. Deighton N, Brennan R, Finn C, Davies HV. Antioxidant properties of domesticated and wild Rubus species. *J. Sci. Food Agric.* 2000;80:1307–1313.

32. Gazzani G, Papetti A, Massolini G, Daglia M. Anti- and prooxidant activity of water soluble components of some common diet vegetables and the effect of thermal treatment. *J. Agric. Food Chem.* 1998;46:4118–4122.

33. Nollet LML, Toldra F. *Handbook of Analysis of Edible Animal By-Products*; CRC Press: Gent, Belgium, 2011; p. 471.

34. Almajano MP, Gordon MH. Synergistic effect of BSA on antioxidant activities in model food emulsions. *Am. Oil Chem. Soc.* 2004;81:275–280.

35. Rampon V, Lethuaut L, Mouhous-Riou N, Genot C. Interface characterization and aging of bovine serum albumin stabilized oil-in-water emulsions as revealed by front-surface fluorescence. *J. Agric. Food Chem.* 2001;49:4046–4051.

36. Frankel EN, Huang SW, Aeschbach R, Prior E. Antioxidant activity of a rosemary extract and its constituents, carnosic acid, carnosol, and rosmarinic acid, in bulk oil and oil-in-water emulsion. *J. Agric. Food Chem.* 1996;44:131–135.

37. Skowyra M, Falguera V, Gallego G, Peiró S, Almajano MP. Antioxidant properties of aqueous and ethanolic extracts of tara (*Caesalpinia spinosa*) pods *in vitro* and in model food emulsions. *J. Sci. Food Agric.* 2013;94:911–918.

38. Sørensen ADM, Haahr AM, Becker EM, Skibsted LH, Bergenståhl B, Nilsson L, Jacobsen C. Interactions between iron, phenolic compounds, emulsifiers, and pH in omega-3-enriched oil-in-water emulsions. *J. Agric. Food Chem.* 2008;56:1740–1750.

39. Donnelly JL, Decker EA, McClements DJ. Iron-catalyzed oxidation of menhaden oil as affected by emulsifiers. *J. Food Sci.* 1998;63:997–1000.

40. Mancuso JR, McClements DJ, Decker EA. Ability of iron to promote surfactant peroxide decomposition and oxidize alpha-tocopherol. *J. Agric. Food Chem.* 1999;47: 4146–4149.

41. Mancuso JR, McClements DJ, Decker EA. The effects of surfactant type, pH, and chelators on the oxidation of salmon oil-in-water emulsions. *J. Agric. Food Chem.* 1999;47:4112–4116.

42. Pangloli P, Melton SL, Collins JL, Penfield MP, Saxton AM. Flavor and storage stability of potato chips fried in cottonseed and sunflower oils and palm olein/sunflower oil blends. *J. Food Sci.* 2002;67:97–103.

43. Wu QX, Li Y, Shi YP. Antioxidant phenolic glucosides from Gentiana piasezkii. *J. Asian Nat. Prod. Res.* 2006;8:391–396.

44. Citová I, Ganzera M, Stuppner H, Solich P. Determination of gentisin, isogentisin, and amarogentin in *Gentiana Lutea* L. by capillary electrophoresis. *J. Sep. Sci.* 2008;31:195–200.

45. Skrzypczak L, Wesolowska M, Bajaj YPS. *Medicinal and Aromatic Plants IV*; Springer Verlag: Berlin, Germany, 1993; Volume 21, pp. 172–186.

46. Ando H, Hirai Y, Fujii M, Hori Y, Fukumura M, Niiho Y, Nakajima Y, Shibata T, Toriizuka K, Ida Y. The chemical constituents of fresh *Gentian* root. *J. Nat. Med.* 2007;61:269–279.

47. Hayashi T, Minamiyama Y, Miura T, Yamagishi T, Kaneshina H. Cultivation of *Gentiana Lutea* and chemical evaluation evaluation of gentiana radix. *Hokkaidoritsu eisei Kenkyushoho* 1990;40:103–106.

48. Phoboo S, Pinto MDS, Barbosa ACL, Sarkar D, Bhowmik PC, Jha PK, Shetty K. Phenolic-linked biochemical rationale for the anti-diabetic properties of *Swertia chirayita* (Roxb. ex Flem.) Karst. *Phytother. Res.* 2013;27:227–235.

49. Oku H, Ogawa Y, Iwaoka E, Ishiguro K. Allergy-preventive effects of chlorogenic acid and iridoid derivatives from flower buds of *Lonicera japonica*. *Biol. Pharm. Bull.* 2011;34;1330–1333.

3.4. The Effect of *Convolvulus arvensis* Dried Extract as a Potential Antioxidant in Food Models

Abstract

In this study, the antioxidant activity of the Convolvulus arvensis Linn (CA) ethanol extract has been evaluated by different ways. The antioxidant activity of the extract assessed by 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical cation, the oxygen radical absorbance capacity (ORAC) and the ferric reducing antioxidant power (FRAP) was 1.62 mmol Trolox equivalents (TE)/g DW, 1.71 mmol TE/g DW and 2.11 mmol TE/g DW, respectively. CA ethanol extract exhibited scavenging activity against the methoxy radical initiated by the Fenton reaction and measured by Electron Paramagnetic Resonance (EPR). The antioxidant effects of lyophilised CA measured in beef patties containing 0.1% and 0.3% (w/w) CA stored in modified atmosphere packaging (MAP) (80% O_2 and 20% CO_2) was determined. A preliminary study of gelatine based film containing CA showed a strong antioxidant effect in preventing the degradation of lipid in muscle food. Thus, the present results indicate that CA extract can be used as a natural food antioxidant.

Keywords:

Convolvulus arvensis; lipid oxidation; active packaging film; antioxidant activity

3.4.1 Introduction

Free radicals produced in the human body result from natural biochemical reactions and, together with external attacks due to stress, smoke and unbalanced diets, among other factors, could cause an imbalance between oxidants and antioxidants. For this reason, it is necessary to supplement the diet with antioxidant based food. This excess of radicals is associated with aging and many diseases such as heart problems, diabetes, neurodegenerative disorder and cancers. Previous studies indicate that the consumption of plant foods rich in antioxidants is beneficial for health and helps to prevent degenerative processes which contribute to many diseases[1-3]. Due to the increasing awareness of the benefits of consuming healthy food, many food companies are using antioxidants as an alternative approach, instead of using synthetic preservatives which at high doses may have toxic effects on the consumer.

Natural antioxidants are compounds, generally from plants, that are used as food additives with the aim of inhibiting oxidation of the product[4]. Thus, the use of natural antioxidants as preservatives to maintain quality and nutritional traits is increasingly widespread, mainly in food that contains high levels of lipids, such as meat products. Therefore, the incorporation of natural antioxidants such as herbs could be an economical strategy to develop healthier meat products. Moreover, they can improve technological properties, as well as increase the eco-efficiency[5] in the food industry. Besides formulation of food with a natural antioxidant strategy, active packaging is also gaining interest for its potential to provide food quality and safety benefits. The combination of natural preservatives and biodegradable plastic into one food packaging formulation is a promising approach to extending product shelf life[6].

Plants rich in polyphenol constituents possess antioxidant activity by free radical scavenging. For instance, green tea can inhibit lipid peroxidation and chelate transition metals, consequently helping to prevent degenerative diseases. If incorporated into an edible film, it could help to maintain the quality of food products[7].

Convolvulus arvensis Linn (CA) is an annual (or sometimes perennial climber), commonly found as a weed throughout Europe and Asia. This plant is being used for many purposes. The root and the resin are cholagogue, diuretic, laxative and purgative[8]. The flower is laxative, used as a tea infusion and also in treatment of wounds and fever, whereas the leaf can be helpful during the menstrual period[9]. Meanwhile, Meng et al. (2002) showed that the ubiquitous CA extract could be considered as a promising anti-cancer agent, with over 50%

inhibition of tumor growth activity at non-toxic doses[10]. CA also provided an immunostimulant effect when tested on rabbits and turned out to have cytotoxic effects on human cancerous cells[11,12]. In a preliminary study, Thrakal et al. (2010) reported the antioxidant activity of CA extract using the DPPH method, nitric oxide scavenging activity and the reducing power assay[13]. Furthermore, the CA extract showed abundant traces of phenolic compounds including *p*-hydrobenzoic acid, syringic acid, vanillin, benzoic acid and ferulic acid[14]. This high content of phenolic compounds may allow it to serve as an antioxidant source for the food industry. However, the antioxidant activity of the CA extract towards lipid oxidation has not been fully determined yet. Thus, our goals were (1) to evaluate the antioxidant activity of CA using *in vitro* assays including FRAP, TEAC, ORAC and EPR scavenging activity and (2) to demonstrate the ability of CA extract to inhibit lipid deterioration in beef meat, by adding the dry extract directly in the patty composition or in the formulation with active packaging. One of the components in CA is an alkaloid, which is a compound that exhibits anti-cancer activity but may display toxic effects in the host at high doses. Therefore, the extraction of CA has been carried out according to the method described by Meng et al. (2002) to reduce the presence of alkaloid in the extract before adding the lyophilized extract directly into the beef[10].

3.4.2 Materials and Methods

Materials

Commercial dried CA was kindly supplied by Pàmies Hortícoles (Balaguer, Spain), a registered herbal company. All reagents and solvents used were of analytical grade and obtained from Panreac (Barcelona, Spain) and Sigma Aldrich (Gillingham, England).

Extraction of CA Extract

Dried roots of CA were finely ground using a standard kitchen food processor. Ground CA was extracted in three different ways: (1) with 50:50 (v/v) ethanol:water; (2) with 75:25 (v/v) ethanol:water and (3) with 90:10 (v/v) ethanol:water, always in the ratio 1:30 (w/v). The extractions were performed at 4 °C ± 1 °C for 24 h, in the dark with constant

stirring. The extract solutions of CA were recovered by filtration using Whatman Filter paper, 0.45 µm (Whatman, GE healthcare, Wauwatosa, WI, USA). Part of the supernatant was taken for subsequent use to determine the antiradical capacity. The volume of the remaining supernatant was measured and the excess of ethanol was removed under vacuum using a rotary evaporator (Buchi Re111, Switzerland) and kept frozen at −80 °C for 24 h. All extracts were dried in a freeze dryer (Unicryo MC2L −60 °C, Germany) under vacuum at −60 °C for three days to remove moisture. Finally, lyophilised CA was weighed to determine the soluble solids concentration (g/L) as described by Zhang et al. (2007)[15].

Determination of the Total Phenolic Content (TPC)

The Folin-Ciocalteu method was used to determine the total phenolic content (TPC) as reported by Santas et al. (2008)[16].

Determination of Free Radical Scavenging Activity Assays

a. *In-Vitro* Antioxidant Capacity Determination

Three different methods were used for the evaluation of the antioxidant activity of the extracts: 2,2′-azino-bis-(3-ethylbenzthiazoline)-6-sulphonic acid TEAC assay[17], Oxygen Radical Absorbance Capacity (ORAC) assay[18] and Ferric Reducing Antioxidant Power (FRAP) method[19]. Results were expressed as µM of Trolox equivalent (TE) per gram of dry weight of plant (DW).

Electron Paramagnetic Resonance (EPR) Spectroscopy Radical Scavenging Assay

EPR radical scavenging activity was measured following the method described by Azman et al. (2014)[20]. The extraction was executed in MeOH in 1:10 (w/v) ratio and the soluble concentration of CA was determined according to the procedure above. The spin-trapping reaction mixture consisted of 100 µL of DMPO (35 mM); 50 µL of H_2O_2 (10 mM); 50 µL CA extract at different concentrations or 50 µL of ferulic acid used as reference (0–20 g/L) or 50 µL of pure MeOH used as a control; and, finally, 50 µL of $FeSO_4$ (2 mM), added

in this order. The final solutions (125 μL) were passed through a narrow (inside diameter = 2 mm) quartz tube and introduced into the cavity of the EPR spectrometer. The spectrum was recorded 10 min after the addition of the $FeSO_4$ solution, when the radical adduct signal is greatest.

X-band EPR spectra were recorded with a Bruker EMX-Plus 10/12 spectrometer under the following conditions: microwave frequency, 9.8762 GHz; microwave power, 30.27 mW; center field, 3522.7 G; sweep width, 100 G; receiver gain, 5.02×10^4; modulation frequency, 100 kHz; modulation amplitude, 1.86 G; time constant, 40.96 ms; conversion time, 203.0 ms.

Determination of Antioxidant Activity in Food Model

a. Preparation of Beef Patties

The meat consisted of flank of beef provided by "Embutidos La Masia", Barcelona. It was collected seven days after slaughter to allow it to mature and was kept at approximately −20 °C for further treatment. The extraction of CA was carried out according to the method used by Meng et al. (2002) to remove alkaloid compounds[10]. Fat and joint tissues were trimmed off lean meat (2000 g) and the meat was minced through 8 mm industrial plates. Then, the minced meat was divided into four batches and mixed with 1.5% of NaCl and either (i) control (no addition), (ii) 0.1% BHT, (iii) 0.1% lyophilised CA, (iv) 0.3% lyophilised CA. All batches were mixed vigorously for 2 min to attain an even distribution of additives throughout the meat. Each sample was moulded into smaller portions (about 20 g each), stuffed and packed with polystyrene B5-37 (Aerpack) trays and placed in BB4L bags (Cryovac) of low gas permeability (8–12 $cm^3 \cdot m^{-2}$ per 24 h). The air in the packaged trays was flushed with 80:20 (v/v) O_2:CO_2 by EAP20 mixture (Carburos Metalicos, Barcelona). Samples were stored in the dark at 4 °C ± 2 °C for 10 days and the samples were analysed for oxidation by thiobarbituric acid reactive substances (TBARS) method, % metmyoglobin, colour, pH and microbial quality. Every measurement was carried out in triplicate each day for 10 days (except for microbiological analysis which was done every three days).

b. TBARS Assay

The TBARS method was used to measure the extent of lipid oxidation over the storage period as described by Grau et al. (2000)[21]. Samples (1 g) were weighed in a tube and mixed with 3 g/L aqueous EDTA. Then, the sample was immediately mixed with 5 mL of thiobarbituric acid reagent using an Ultra-Turrax (IKA, Germany); at 32,000 rpm speed, for 2 min. All procedures were carried out in the dark and all samples were kept in ice. The mixture was incubated at 97 ± 1 °C in hot water for 10 min and shaken for 1 min during the process to form a homogeneous mixture. The liquid sample was recovered by filtration (Whatman Filter paper, 0.45 μm), and then it was cooled for 10 min. The absorbance value of each sample was measured at 531 nm using a spectrophotometer. The TBARS value was calculated from a malonaldehyde (MDA) standard curve prepared with 1,1,3,3 tetraethoxypropane and analysed by linear regression. All results were reported in mg malonaldehyde per kg of sample (mg MDA/kg sample).

c. Colour Measurement

Objective measurements of colour were performed using a CR 400 colorimeter (Minolta, Osaka, Japan). Each patty was cut and the colour of the slices was measured three times at each point. A portable colorimeter with the settings: pulsed xenon arc lamp, 0° viewing angle geometry and aperture size 8 mm, was used to measure meat colour in the CIELAB space (Lightness, L*; redness, a*; yellowness, b* (CIE, 1978). Before each series of measurements, the instrument was calibrated using a white ceramic tile.

d. Percentage of Metmyoglobin

The metmyoglobin method was based on that developed by Xu et al. (2010)[22]. Five grams of beef patties were homogenized with 25 mL of ice-cold 0.04 M phosphate buffer (pH 6.8) for 15 s using a homogenizer (Ultra-Turrax, IKA, Germany), which was set at speed setting 2 (18,000 rpm). The homogenised patty was allowed to stand at 4 °C for 1 h and centrifuged at 4500 g for 20 min at 4 °C using a high-speed freezing centrifuge (GI-20G, Anke, Shanghai, China). The absorbance of the filtered supernatant was read at 572, 565, 545, and 525 nm with a spectrometer (Fluostar Omega, BMG Labtech, Germany). The percentage of metmyoglobin was determined using the formula: $\text{MetMb (\%)} = [-2.514 (A572/A525) + 0.777 (A565/A525) + 0.8 (A545/A525) + 1.098] \times 100$

Development of Gelatin-Film with Antioxidant Coating

The fabrication of gelatin based film with antioxidant coating was adapted and characterized from Bodini et al. (2013)[23]. While the filmogenic solution was cooled after the solubilization of sorbitol, 0.75% (w/w) of CA extract / gelatin and 0.1% (w/w) BHT/gelatin were added.

Statistical Analysis

A one-way analysis of variance (ANOVA) was performed using Minitab 16 software program (Minitab Pty Ltd., Sydney, Australia) ($\alpha = 0.05$). The results were presented as mean values ($n \geq 3$).

3.4.3 Results and Discussion

Analysis of Total Polyphenols and Free Radical Activity Assays

On average, a higher weight of soluble solids was extracted from CA with 50% ethanol than with 75% and 90% of ethanol. The use of ethanol as extraction solvent is due to the fact that the solvent is recognized as a GRAS (Generally Recognized as Safe) component which can be safely used for applications in the food industry[24]. Ethanol also turned out to be effective in the extraction of flavonoids and their glycosides, catechols and tannins from raw plant materials. Generally, CA extracted with 50% ethanol showed higher phenolic content and antioxidant activity values in ORAC, FRAP and TEAC. Our results showed (Table 1) that the total phenolic content correlated with the antioxidant activity determined by the assays. Nevertheless, the values obtained in the ORAC assay were higher than the ones in the FRAP and TEAC assays, which also showed the extract scavenging activity against peroxy radicals ($OOH^{\bullet}$) generated in the assay.

Table 1: Soluble solids concentration, total phenolic content (TPC) and antioxidant activity of *Convolvulus arvensis Linn* (CA) extract.

Activity *Convolvulus arvensis*	Extraction Solvent		
	50:50 EtOH:H_2O	75:25 EtOH:H_2O	90:10 EtOH:H_2O
Soluble concentration (g/L)	13.76 ± 0.05	13.61 ± 0.02	11.43 ± 0.05
Total phenolic content (g GAE/g DW)	13.0 ± 0.05	12.1 ± 0.03	9.9 ± 0.02
FRAP (mmol of TE/g DW)	1.62 ± 0.02	1.51 ± 0.06	0.98 ± 0.01
TEAC (mmol of TE/g DW)	1.71 ± 0.01	1.68 ± 0.01	1.41 ± 0.04
ORAC (mmol of TE/g DW)	2.11 ± 0.05	2.05 ± 0.05	1.71 ± 0.03

* Mean value n = 3.The standard deviation for each assay is less than 5%. Gallic Acid Equivalent (GAE), Trolox Equivalent (TE), Dry Weight (DW).

Total phenolic content reported for the plant extract with ethyl acetate turned out to be higher than our present results with 244 mg GAE/g DW[24]. The presence of compounds with antioxidant potential in the ethanol extract (Table 1) was revealed in the measurement of total antioxidant capacity in this study. In previous studies, the antioxidant activity of CA has been analyzed using the DPPH method, nitric oxide scavenging activity and reducing power assay applied to both methanol and ethyl acetate solvent extracts[13,25]. To the best of our knowledge, this is the first report of the antioxidant activity of CA extracts assessed using the TEAC, ORAC and FRAP methods.

EPR Scavenging Radical Assay

The EPR radical scavenging method has been developed by Azman et al. (2014) to evaluate the concentration of free methoxy radicals ($CH_3O^{\bullet}$) generated in the Fenton reaction with the CA extract[20]. Figure 1 below shows the decreasing signal of EPR with the increase of CA extract concentration.

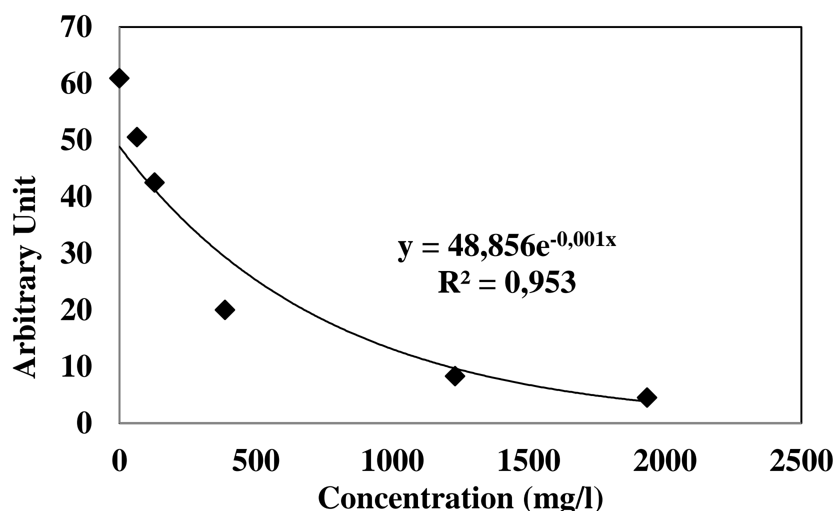


Figure 1. Antioxidant activity determined by the Electron paramagnetic resonance (EPR) spectrum of the radical adduct DMPO-OCH_3 generated from a solution of H_2O_2 (2 mM) and $FeSO_4$ (0.04 mM) with DMPO (14 mM) as spin trap in MeOH as solvent. The EPR signal decreases with the higher antioxidant activity.

The free radical scavenging activity of CA extracts was investigated against methoxy ($CH_3O^\bullet$) radical by a competitive method in the presence of DMPO as spin trap, using EPR spectroscopy. $CH_3O^\bullet$ was generated according to the Fenton procedure with a relatively short half-life that was identified by EPR because of its ability to form a stable nitroxide adduct with DMPO, DMPO-OCH_3 (hyperfine splitting constants, a_N = 13.9 G and a_H = 8.3 G). This stable DMPO-OCH_3 compound can be detected by the double integration value of the signal from EPR. The presence of CA extract at different concentrations may compete with the spin trap in the scavenging of methoxy radicals. Thus, the effect reduces the amount of radical adducts and, accordingly, reduces the intensity of the EPR signal. The best fitting with intensity of EPR signal was shown as an exponential function (Figure 1) that, if concentration values are in g/L, corresponds to Equation (1):

$$y = 48.856\ e^{-0.001\,x};\ R^2 = 0.953 \qquad (1)$$

The graph indicates that the exponential value of the signal of the spectrum decreased as the amount of CA increased. This study confirmed that the scavenging activity of the *Convolvulus arvensis* extracts containing polyphenol constituents could be measured by the decrease of the intensity of the spectral bands of the adduct DMPO-OCH_3 in the EPR spectrum with the amount of antioxidant.

Antioxidant Activity in Model Food

a. Colour and % Metmyoglobin

Meat colour is one of the most important traits that reflect the meat freshness and quality for consumers. The colour parameters representing lightness (L*), redness (a*), and yellowness (b*) are shown in Table 2. Generally, the value of colour (L*, a* and b*) decreased as the storage time increased. Initial mean lightness (CIE L*) was 38.68 ± 0.87, and control sample showed the lowest value of L* at the end of 10 days storage. There are marginally differences in L* with all samples throughout storage times. The slight change of L* value in meat storage was addressed by few authors[26,27]. The decrease of L* value indicates that a darkening developed, which may be due to the Maillard reaction or the effect of moisture content, which influences lightness values[28,29].

A reduction of the a* value was experienced by all samples in 10 days' storage ($p < 0.05$), indicating that a decrease in redness occurred in the meat. The 0.1% BHT displayed the highest value of a* during three days' storage and declined gradually afterwards ($p < 0.05$). This finding was expected due to the role of BHT as a synthetic antioxidant which is used to retain colour and delay lipid oxidation in the meat [30]. The redness of 0.3% CA was maintained around a value of 7 during the eight days before the colour faded rapidly in 10 days' storage ($p > 0.05$). At the end of storage, 0.3% CA showed the highest a* value followed by 0.1% CA ($p < 0.05$) and 0.1% BHT and control exhibited a low value with no significant difference between both samples ($p > 0.05$). Many features contributed to the red colour in the meat such as the influence of salt and oxygen composition that enhanced the red colour of beef patties[31,32]. The samples had an initial yellowness (b*) value of 7.42 ion that enhanced the red in both samples (eight days before $p > 0.05$). In general, no significant difference ($p > 0.05$) was observed in b* values in all samples throughout storage. The present findings seem to be consistent with other research which found that yellowness in meat patties is not influenced by storage time and packaging conditions[26,33].

Table 2. Effect of CA extract and BHT on instrumental colour value (L*, a*, b*) of beef patties during 10 days of refrigerated storage at 4 °C. (Mean ± SE).

Assay	Sample	Days of Storages					
		0	2	4	6	8	10
L*	Control	38.68 ± 0.87 ^{a1}	38.68 ± 1.50 ^{a,1}	37.89 ± 0.32 ^{b,3}	37.10 ± 1.23 ^{b,2}	36.23 ± 0.45 ^{c,2}	35.61 ± 2.22 ^{d,1}
	0.1% BHT	38.68 ± 0.87 ^{a1}	39.06 ± 1.08 ^{b,2}	38.25 ± 0.97 ^{a,2}	38.43 ± 1.06 ^{a,1}	37.09 ± 1.19 ^{c,1}	36.18 ± 0.46 ^{c,2}
	0.1% CA	38.68 ± 0.87 ^{a1}	38.60 ± 1.05 ^{a,1}	39.26 ± 1.46 ^{b,1}	38.63 ± 0.55 ^{a,1}	37.11 ± 1.02 ^{c,1}	37.06 ± 1.22 ^{c,3}
	0.3 % CA	38.68 ± 0.87 ^{a1}	39.94 ± 0.71 ^{b,2}	39.79 ± 1.23 ^{b,1}	38.25 ± 1.40 ^{a,1}	38.91 ± 1.47 ^{a,3}	38.84 ± 1.13 ^{a,4}
a*	Control	7.49 ± 0.27 ^{a1}	7.77 ± 0.29 ^{a,1}	6.54 ± 0.33 ^{b,1}	6.27 ± 0.16 ^{b,2}	4.71 ± 0.02 ^{c,1}	2.09 ± 0.01 ^{d,1}
	0.1% BHT	7.49 ± 0.27 ^{a1}	8.18 ± 0.42 ^{b,2}	9.28 ± 0.28 ^{c,2}	7.05 ± 0.31 ^{a,1}	6.36 ± 0.37 ^{d,2}	2.87 ± 0.01 ^{e,1}
	0.1% CA	7.49 ± 0.27 ^{a1}	7.61 ± 0.33 ^{a,1}	5.57 ± 0.26 ^{b,3}	6.25 ± 0.19 ^{c,2}	6.60 ± 0.33 ^{c,2}	3.31 ± 0.02 ^{d,2}
	0.3 % CA	7.49 ± 0.27 ^{a1}	7.64 ± 0.21 ^{a,1}	7.20 ± 0.47 ^{a,4}	7.50 ± 0.20 ^{a,1}	7.61 ± 0.37 ^{a,3}	4.08 ± 0.01 ^{b,3}
b*	Control	7.42 ± 0.32 ^{a1}	4.86 ± 0.01 ^{b,1}	7.68 ± 0.36 ^{a,1}	8.55 ± 0.19 ^{c,1}	9.95 ± 0.21 ^{d,1}	6.77 ± 0.02 ^{e,1}
	0.1% BHT	7.42 ± 0.32 ^{a1}	6.68 ± 0.16 ^{b,2}	8.40 ± 0.27 ^{c,1}	8.39 ± 0.37 ^{c,1}	8.38 ± 0.24 ^{c,2}	6.10 ± 0.01 ^{d,1}
	0.1% CA	7.42 ± 0.32 ^{a1}	8.00 ± 0.37 ^{b,3}	8.19 ± 0.33 ^{b,1}	5.17 ± 0.13 ^{c,2}	7.49 ± 0.07 ^{a,3}	4.35 ± 0.09 ^{d,2}
	0.3 % CA	7.42 ± 0.32 ^{a1}	7.14 ± 0.49 ^{a,4}	7.59 ± 0.29 ^{a,2}	7.01 ± 0.21 ^{a,3}	7.99 ± 0.27 ^{a,3}	3.25 ± 0.01 ^{b,3}

Control: 1.5% salt (w/w); 0.1% BHT: 1.5% salt with 0.1% BHT (w/w); 0.1% CA: 1.5% salt with 0.1% CA (w/w) 0.3% CA: 1.5% salt with 0.3% CA (w/w). $^{a–d}$: Means within a row with different letters are significantly different ($p < 0.05$). $^{1–4}$: For each attribute, means within a column with different number are significantly different ($p < 0.05$). Mean value $n = 6$ and the standard deviation for each assay is less than 5%.

Table 3. Effects of CA extract and BHT on metmyoglobin changes in beef patties during 10 days of refrigerated storage at 4 °C. (Mean ± SE).

Assay	Sample	Day of Storages					
		0	2	4	6	8	10
% Metmyoglobin	Control	23.38 ± 0.46 [1]	29.19 ± 0.71 [2]	37.56 ± 1.31 [2]	47.84 ± 1.21 [1]	53.9 ± 1.16 [1]	60.03 ± 2.82 [2]
	0.1% BHT	23.38 ± 0.46 [1]	25.69 ± 1.04 [4]	29.98 ± 0.81 [1]	37.5 ± 1.85 [2]	45.7 ± 1.53 [2]	57.6 ± 1.24 [1]
	0.1% CA	23.38 ± 0.46 [1]	27.96 ± 0.33 [1]	33.48 ± 0.79 [3]	44.81 ± 1.29 [3]	48.7 ± 1.67 [3]	57.1 ± 1.18 [1]
	0.3 % CA	23.38 ± 0.46 [1]	28.91 ± 0.81 [1,2]	30.71 ± 0.29 [1]	33.37 ± 0.94 [4]	40.1 ± 1.53 [4]	50.78 ± 1.56 [3]

Control: 1.5% salt (w/w); 0.1% BHT: 1.5% salt with 0.1% BHT (w/w); 0.1% CA: 1.5% salt with 0.1% CA (w/w) 0.3% CA: 1.5% salt with 0.3% CA (w/w). All samples values are significantly different throughout the storage time ($p < 0.05$) [1–4]: Means within a column with different numbers are significantly different ($p < 0.05$). Mean value $n = 6$ and the standard deviation for each assay is less than 5%.

The effect of CA extracts and BHT on relative MetMb percentage in beef patties are presented in Table 3. A significant correlation between MetMb (%) and the instrumental colour features was reported previously[22]. The MetMb percentage increased as the storage time increased throughout the 10 days' refrigeration, whereas the control showed the highest MetMb compared to all samples. The treated groups of CA extract and BHT had lower ($p < 0.05$) proportions of MetMb compared to the control at the end of storage. The acceleration of colour deterioration and lipid oxidation depended on many causes, including storage time, type of packaging and test system. Free radicals produced by lipid oxidation in meat are susceptible to initiating the reaction of oxidizing oxymyoglobin (red colour) to metmyoglobin (brown colour) which results in the discolouration of meat during storage. Previous research has indicated a relationship between lipid oxidation and myoglobin oxidation or discolouration in meat products[22,24]. A sufficient amount of antioxidant in the sample can delay the formation of metmyoglobin. The scavenging ability of samples treated with antioxidant can reduce the oxidation of metmyoglobin acting as scavengers of hydroxyl radicals produced from oxidation of oxymyoglobin. The 0.3% of CA extract displayed the lowest metmyoglobin percentage compared to all samples, and the change of % metmyoglobin was inversely proportional to the value of redness (a*).

b. TBARS Analysis in Beef Patties

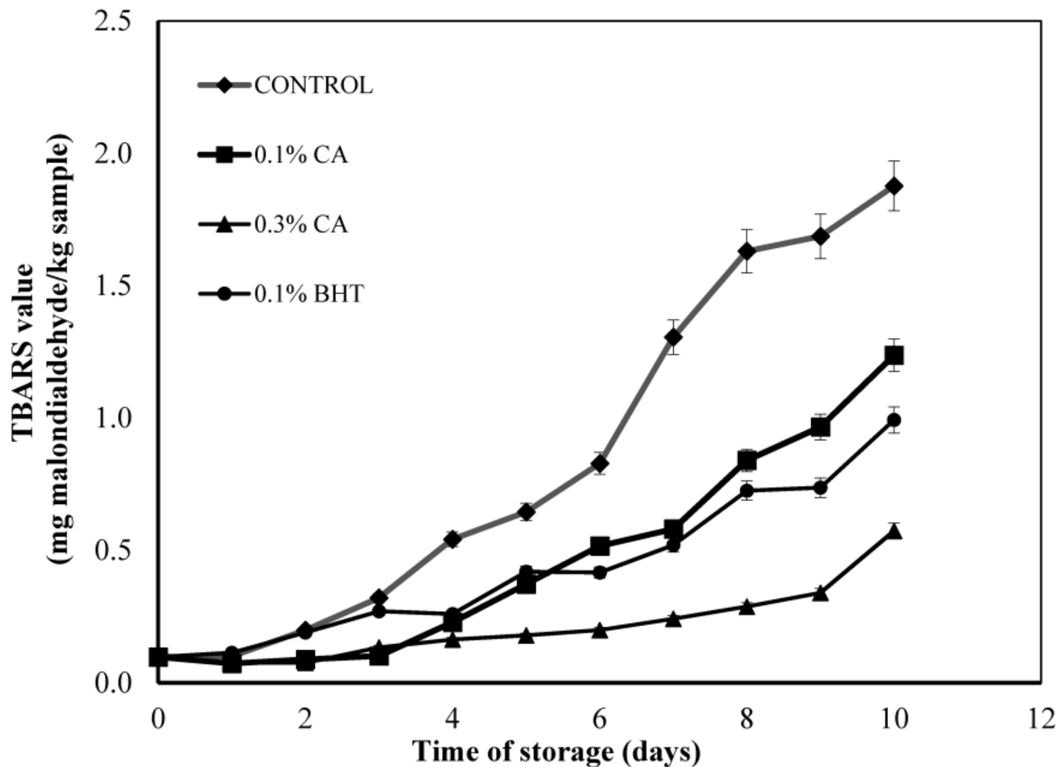


Figure 2. Changes in TBARS values (mg malondialdehyde/kg sample) of control and sample containing different concentrations (0.1% and 0.3% w/w) of CA extract in MAP atmosphere during 10 days storage at 4 ± 1 °C without light. Each sample was measured in triplicate and the average standard deviation for each sample was less than 5%.

In general, the levels of lipid oxidation in beef patties increased over time and the values followed the order: 0.3% CA < 0.1% BHT < 0.1% CA < Control (Figure 2). The presence of a controlled atmosphere with high oxygen packaging (MAP) resulted in higher TBARS values and increased the oxidation rate in muscle food[32,35]. No statistical difference was observed between 0.1% BHT and 0.1% CA on any of the storage days However, the TBARS values of both samples showed significant differences compared to those of the control samples ($p < 0.05$). From seven days onwards, the control reached the highest TBARS values of all samples, with values greater than 1.2 mg malonaldehyde/kg sample. The levels of lipid oxidation were the lowest in 0.3% CA in beef patties throughout storage and significantly lower than for all other samples. The oxidation rate of meat patties was more reduced for a higher concentration of CA extract, as shown by comparison of the rates for 0.1% and 0.3% addition. The 0.1% BHT was

added for comparison with the natural antioxidant bearing in mind the FDA guidelines for using BHT is ≤200 ppm in meat products. The effect of CA extract on lipid oxidation in meat has never been reported. The active properties of CA reported by Hegab and Ghareib (2010)[14] have been attributed to various phenolic acids such as ferulic acid, cinnamic acid and *p*-coumaric acid. The antioxidant activity of phenolic compounds is closely related to the hydroxyl group linked to the aromatic ring which is capable of donating hydrogen atoms with electrons and neutralizing free radicals. This mechanism blocks further degradation by oxidation to form MDA, which can be measured by the TBARS method[36]. This study confirmed the potential of CA extract to inhibit lipid degradation in beef patties.

c. TBARS Analysis in Meat under Active Packaging

The TBARS index (Figure 3) revealed that the coating of beef patties with edible films enriched with antioxidants lowered the oxidation rate during 17 days' storage. By comparison, the gelatin film without any added antioxidants did not display any protective effect. Lipid oxidation with respect to TBARS values of control, meat patties sample and those wrapped with CA and BHT incorporated film showed a significantly different TBARS value ($p < 0.05$) than the control sample. This result suggested that lipid oxidation in meat samples could be minimized by the use of a gelatin film containing CA probably due to the antioxidant activity of the CA extract. However, BHT and CA coated in gelatin film did not show any significant difference between the values for the different periods of storage.

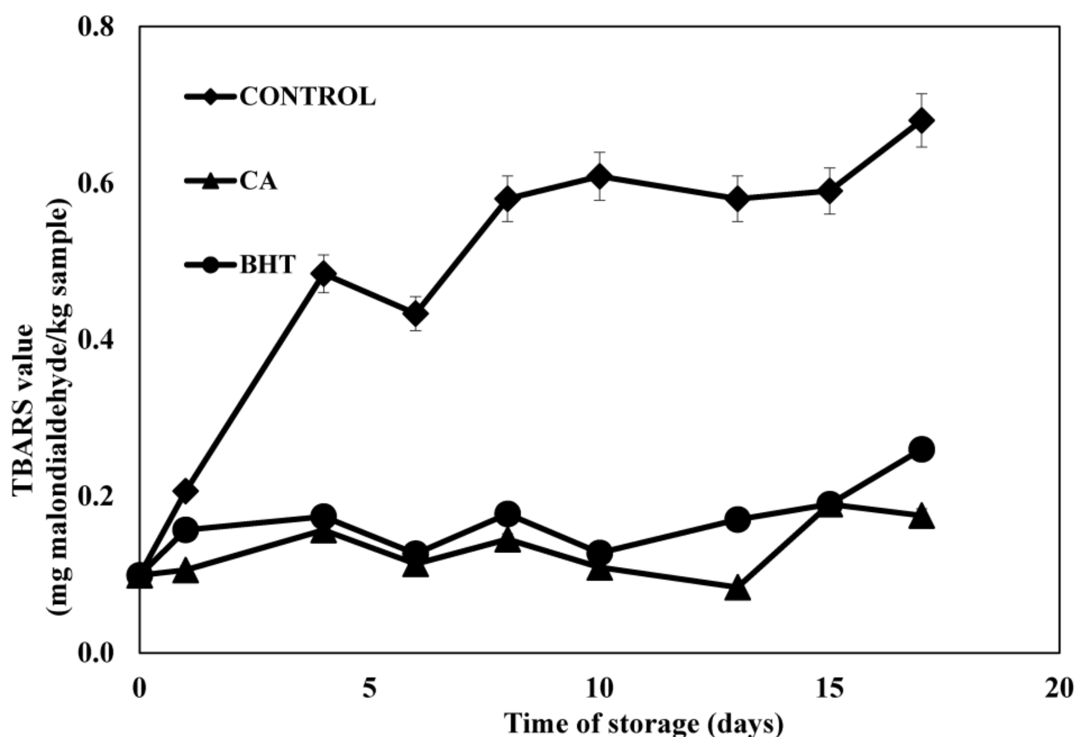


Figure 3. Changes in TBARS values (mg malondialdehyde/kg sample) of control and sample containing BHT and CA extract in MAP atmosphere during 17 days' storage at 4 ± 1 °C without light. Each sample was measured in triplicate and the average standard deviation for each sample was less than 5%.

Duthie et al. (2013) demonstrated the presence of phenolic acids measured using LC-MS in chicken patties mixed with vegetable powders including ferulic acid, *p*-hydrobenzoic acid, *p*-coumaric acid, caffeic acid and cinnamic acid[37]. In reviewing the literature, CA contained a great amount of phenolic compounds that may be responsible for its strong antioxidant activity in many assays. The constituents included *p*-hydroxybenzoic acid, syringic acid, vanillin, benzoic acid, ferulic acid found by Elzaawely and Tawata (2012)[25]. HPLC analysis done by Hegab and Ghareib (2010) showed traces of eight phenolic constituents including pyrogallic acid, protocatechuic acid, resorcinol, chologenic acid, caffeic acid, salicylic acid, *p*-coumaric acid and cinnamic acid[14]. These compounds lead to many pharmacological benefits to human health. Benzoic acid and its derivatives showed antimicrobial potential[38] while gallic acid and caffeic acid showed 50% inhibitory effects on cancer cell proliferation[39]. *p*-coumaric, ferulic acid and cinnamic acid and their derivatives bring many pharmacological benefits to humans including, anticancer and antioxidant effects [40,41]. Moreover, many constituents detected in the CA extract

correlated significantly with antioxidant activity measured by ORAC and TEAC assays and have played an important role in the detoxification of endogenous compounds in humans[42].

3.4.4 Conclusions

The CA extract showed an excellent antioxidant activity in 50% aqueous ethanol measured by FRAP, TEAC and ORAC assays. This is also the first time that the radical scavenging activity has been evaluated in a CA extract against methoxy radical generated in the Fenton Reaction assessed by EPR.

The CA extract also showed a protective effect against lipid degradation in the muscle food model. Lyophilised CA (0.1% and 0.3% w/w) can be applied as an antioxidant in meat patties. It showed inhibition of lipid oxidation in MAP. 0.3% of CA retained meat redness and browning colour measured by the metmyoglobin assay which was much better than the control ($p < 0.05$) during 10 days' storage. A preliminary study of gelatin based film coated with CA showed there was a significant delay in the lipid degradation in beef ($p < 0.05$). Therefore, this study confirmed that CA could be used by the food industry as a source of antioxidants.

References

1. Alvarez-Suarez JM, Giampieri F, González-Paramás AM, Damiani E, Astolfi P, Martinez-Sanchez G, Bompadre S, Quiles JL, Santos-Buelga C, Battino M. Phenolics from monofloral honeys protect human erythrocyte membranes against oxidative damage. *Food Chem. Toxicol.* 2012;50:1508–16.

2. Zhao G, Yin Z, Dong J. Antiviral efficacy against hepatitis B virus replication of oleuropein isolated from Jasminum officinale L. var. grandiflorum. *J. Ethnopharmacol.* 2009;125:265–8.

3. Chang-Liao WL, Chien CF, Lin L, Tsai TH. Isolation of gentiopicroside from Gentianae Radix and its pharmacokinetics on liver ischemia/reperfusion rats. *J. Ethnopharmacol.* 2012;141:668–73.

4. Halliwell B. Antioxidants in human health and disease. *Annu. Rev. Nutr.* 1996;16:33–50.

5. Martín-Sánchez AM, Navarro C, Pérez-Álvarez JA, Kuri V. Alternatives for Efficient and Sustainable Production of Surimi : A Review. *Compr. Rev. Food Sci. Food Saf.* 2009;8:359–374.

6. Suppakul P, Miltz J, Sonneveld K, Bigger SW. Active Packaging Technologies with an Emphasis on Antimicrobial Packaging and its Applications. *J. Food Sci.* 2003;68:408–420.

7. Siripatrawan U and Noipha S. Active film from chitosan incorporating green tea extract for shelf life extension of pork sausages. *Food Hydrocoll.* 2012;27:102–108.

8. Chopra RN, Nayar SL, Chopra IC. *Glossary of Indian Medicinal Plants (Including supplement)*. 1986. CSIR. New Delhi, India.

9. Foster S, Duke JA. *A Field Guide of Medicinal Plants*. 1990. Houghton Mifflin Co. New York, USA.

10. Meng XL, Riordan NH, Casciari JJ, Zhu Y, Gonzalez JM, Miranda-Massari JR, Riordan HD. Effect of High Molecular Mass Convolvulus Arvensis Extract on Tumor Growth and Angiogenesis. *Pharmacognosis*. 2002;21:323–328.

11. Ghasemi N and Kohi M. Cytotoxic effect of Convolvulus arvensis extracts on human cancerous cell line. 2008;1520:31–34.

12. Al-Bowait ME, Albokhadaim IF, Homeida AM. Immunostimulant Effect of Binweed (Convolvulus Arvensis) Extract in Rabbit. *Res. J. Pharmacol.* 2010;4:51–54.

13. Thakral J, Borar S, Kalia AN. Antioxidant Potential Fractionation from Methanol Extract of Aerial Parts of Convolvulus arvensis Linn. (Convolvulaceae). *Int. J. Pharm. Sci. Drug Res.* 2010;2:219–223.

14. Hegab MM, Ghareib HR. Methanol Extract Potential of Field Bindweed (Convolvulus arvensis L) for Wheat Growth Enhancement. *Int. J. Bot.* 2010;6:334–342.

15. Skowyra M, Falguera V, Gallego G, Peiró S, Almajano MP. Antioxidant properties of aqueous and ethanolic extracts of tara (Caesalpinia spinosa) pods in vitro and in model food emulsions. *J. Sci. Food Agric.* 2013;94:911-8.

16. Santas J, Carbo R, Gordon M, Almajano MP. Comparison of the antioxidant activity of two Spanish onion varieties. *Food Chem.* 2008;107:1210–1216.

17. Almajano MP, Carbó RL, Jiménez JA, Gordon MH. Antioxidant and antimicrobial activities of tea infusions. *Food Chem.* 2008;108:55–63.

18. Gallego MG, Gordon MH, Segovia FJ, Skowyra M, Almajano MP. Antioxidant properties of three aromatic herbs (rosemary, thyme and lavender) in oil-in-water emulsions. *J. Am. Oil Chem. Soc.* 2013;90:1559–1568.

19. Azman NAM, Peiró S, Fajarí L, Julià L, Almajano MP. Radical scavenging of white tea and its flavonoid constituents by electron paramagnetic resonance (EPR) spectroscopy. *J. Agric. Food Chem.*, 2014;62:5743–8.

20. Elzaawely AA and Tawata S. Antioxidant Activity of Phenolic Rich Fraction Obtained from Convolvulus arvensis L. leaves Grown in Egypt. *Asian J. Crop Sci.* 2012;4:32–40.

21. Grau A, Guardiola F, Boatella J, Barroeta A, Codony R. Measurement of 2-thiobarbituric acid values in dark chicken meat through derivative spectrophotometry: influence of various parameters. *J. Agric. Food Chem.* 2000;48:1155–9.

22. Xu Z, Tang M, Li Y, Liu F, Li X, Dai R. Antioxidant properties of Du-zhong (Eucommia ulmoides Oliv.) extracts and their effects on color stability and lipid oxidation of raw pork patties. *J. Agric. Food Chem.* 2010;58:7289–96.

23. Bodini RB, Sobral PJ, Favaro-Trindade CS, Carvalho RA. Properties of gelatin-based films with added ethanol–propolis extract. *LWT - Food Sci. Technol.* 2013;51:104–110.

24. Kiselova Y, Ivanova D, Chervenkov T, Gerova D, Galunska B, Yankova T. Correlation between the in vitro antioxidant activity and polyphenol content of aqueous extracts from Bulgarian herbs. *Phytother. Res.* 2006;20:961–5.

25. Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid. Med. Cell. Longev.* 2009;2:270–8,.

26. Fernández-Agullóa A, Pereira E, Freire MS, Valentão P, Andrade PB, González-Álvarez J, Pereira JA. Influence of solvent on the antioxidant and antimicrobial properties of walnut (Juglans regia L.) green husk extracts. *Ind. Crops Prod.* 2013;42:126–132.

27. Cachaldora A, García G, Lorenzo JM, García-Fontán MC. Effect of modified atmosphere and vacuum packaging on some quality characteristics and the shelf-life of 'morcilla', a typical cooked blood sausage. *Meat Sci.* 2013;93:220–5.

28. Martín-Sánchez AM, Ciro-Gómez G, Sayas E, Vilella-Esplá J, Ben-Abda J, Pérez-Álvarez JÁ. Date palm by-products as a new ingredient for the meat industry: application to pork liver pâté. *Meat Sci.* 2013;93:880–7.

29. Pérez-Álvarez JA, Fernández-López J. *Color characteristics of meat and poultry analysis, Handbook of processed meats and poultry analysis*. Boca Raton: CRC Press, 2009, pp. 355–373.

30. Sidhu JS. *Date fruits production and processing, Handbook of fruits and fruit processing*, In Y. H. H. Iowa, USA: Blackwell Publishing, 2006, pp. 391–420.

31. Sánchez-Escalante A, Djenane D, Torrescano G, Beltrán J, Roncalés P. The effects of ascorbic acid, taurine, carnosine and rosemary powder on colour and lipid stability of beef patties packaged in modified atmosphere. *Meat Sci.* 2001;58:421–9.

32. Azman NAM, Gordon MH, Skowyra M, Segovia F, Almajano MP. Use of lyophilised and powdered Gentiana lutea root in fresh beef patties stored under different atmospheres. *J. Sci. Food Agric.* 2014. Epub print ahead.

33. Martínez L, Djenane D, Cilla I, Beltrán JA, Roncalés P. Effect of varying oxygen concentrations on the shelf-life of fresh pork sausages packaged in modified atmosphere. *Food Chem.* 2006;94:219–225.

34. Triki M, Herrero AM, Rodríguez-Salas L, Jiménez-Colmenero F, Ruiz-Capillas C. Chilled storage characteristics of low-fat, n-3 PUFA-enriched dry fermented sausage reformulated with a healthy oil combination stabilized in a konjac matrix. *Food Control*. 2013;31:158–165.

35. O'Grady, MN, Monahan F, Brunton NP. Oxymyoglobin Oxidation and Lipid Oxidation in Bovine Muscle—Mechanistic Studies. *J. Food Sci.* 2001;66:386–392.

36. Azman NAM, Segovia F, Martínez-Farré X, Gil E, Almajano MP. Screening of Antioxidant Activity of Gentian Lutea Root and Its Application in Oil-in-Water Emulsions. *Antioxidants*. 2014;3:455–471.

37. Kim SJ, Cho AR, Han J. Antioxidant and antimicrobial activities of leafy green vegetable extracts and their applications to meat product preservation. *Food Control.* 2013;29:112–120.

38. Oloyede GK, Willie IE, Adeeko OO. Synthesis of Mannich bases: 2-(3-Phenylaminopropionyloxy)-benzoic acid and 3-Phenylamino-1-(2,4,6-trimethoxy-phenyl)-propan-1-one, their toxicity, ionization constant, antimicrobial and antioxidant activities. *Food Chem.* 2014;165:515–21.

39. Tao L, Wang S, Zhao Y, Sheng X, Wang A, Zheng S, Lu Y. Phenolcarboxylic acids from medicinal herbs exert anticancer effects through disruption of COX-2 activity. *Phytomedicine*. 2014;21:1473–82.

40. Kiliç I and Yeşiloğlu Y. Spectroscopic studies on the antioxidant activity of p-coumaric acid. *Spectrochim. Acta. A. Mol. Biomol. Spectrosc.* 2013;115:719–24.

41. Nithiyanantham S, Siddhuraju P, Francis GA promising approach to enhance the total phenolic content and antioxidant activity of raw and processed Jatropha curcas L. kernel meal extracts. *Ind. Crops Prod.* 2013;43:261–269.

42. Yeh CT and Yen GC. Effects of phenolic acids on human phenolsulfotransferases in relation to their antioxidant activity. *J. Agric. Food Chem.* 2003;51:1474–9.

43. Duthie G, Campbell F, Bestwick C, Stephen S, Russell W. Antioxidant effectiveness of vegetable powders on the lipid and protein oxidative stability of cooked Turkey meat patties: implications for health. *Nutrients*. 2013;5:1241–52.

3.5. Solvent effect on antioxidant activity and total phenolic content of *Betula Pendula Roth.* and *Convolvulus Arvensis Linn*

Abstract

The potential of using herbal *Betula Pendula Roth.* (BP) and *Convolvulus Arvensis Linn* (CA) as a natural antioxidant for food applications were investigated. Each plant extract was prepared by using pure ethanol, different concentration of ethanol aqueous solutions, including 50% and 75%, 50% methanol aqueous and water. Total phenolic content (TPC) was determined using Folin–Ciocalteau method and antioxidant activity were analyzed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, trolox equivalent antioxidant capacity (TEAC), Oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) respectively. Ethanol extract of CA exhibited the highest TPC and antioxidant activity; however BP showed varies of antioxidant activity value in each assay. The BP and CA exhibit the potential sources of natural antioxidant for food commodities

Keywords:

Solvent effect, Antioxidant activity, *Betula Pendula, Convolvulus Arvesis*, Total Phenolic Content

3.4.1 Introduction

Reactive oxygen species (ROS) is an intermediate product occurs from natural biological combustion in the organism respiration process. Excessive of ROS can lead to cumulative damage in proteins, lipids and DNA called as an oxidative stress in the body[1]. This imbalance mechanism promotes aging processes and various diseases in humans including cancer, neurodegenerative diseases, inflammation, and cardiovascular disease. Thus, the consumption of antioxidant foods is believed to be important to create balance between antioxidant and oxidation process for reducing the production of ROS and resulted to healthier biological system[1,2].

Medicinal plants, fruits and selected herbs are among foods that associated to their natural antioxidant contents. Hence, several methods have been developed to measure the antioxidant activity in plants, including the oxygen radical absorption capacity (ORAC)[3], ferric reducing antioxidant power (FRAP)[4], 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging[5], and trolox equivalent antioxidant capacity (TEAC)[6]. The use of natural plants presents a large source of novel bioactive compounds with different activities. In continuation of our screening programme to search for potential plant, *Betula Pendula Roth.* (BP) and *Convolvulus Arvensis Linn.* are among selected herbal plants to be studied for their potential activities. This study was extended to obtain the best alcohol aqueous solvent for retrieval antioxidant activity in the plant extract.

Betula Pendula Roth. (BP) is also known as *Betula Alba* or *Betula Pubescens*, a species of native birch mostly found throughout northern Europe, Asia, Iceland and Greenland[7]. BP was reported to display several biological effects, including anti-viral, anti-parasitic, anti-bacterial, anti-inflammatory activities and anti-cancer to inhibit growth of cancer cells[8]. It has been demonstrated recently that BP is also inhibit effectively against head and neck squamous carcinoma cells[9,10], leukemia cells and other cells lines[11,12].

Convolvulus arvensis Linn (CA) is a perennial climber commonly found as a weed throughout in the Nile region, the Libyan desert oasis and Sinai[13]. Reviewing from the previous works, it was mentioned in folk medicine that the CA leaves have a purgative activity used in asthma, jaundice and anti-hemorrhagic[14,15]. Some phytochemical studies were carried out on CA

showed the plant contains alkaloids, phenolic compounds, sterols, resin and sugars. Moreover, CA is also believed to have an anticancer effect[15].

Through many researches done to identify the active compounds of BP and CA, these studies are aimed to evaluate the phenolic content (TPC) and antioxidant activities of BP and CA. The study is extended by determining the best solvent extract between concentration of 100%, 75%, 50% (v/v) of ethanol aqueous, water and 50% (v/v) methanol aqueous on their phenol contain and antioxidant activities. Antioxidant activities of the plants were measured by improved method of TEAC[6], FRAP assay[4], ORAC[3] and a modified DPPH assay[5]. Furthermore, we are also estimated the phenol content of these plants using the classical Folin–Ciocalteu reagent[16]. This study proved the potential of BP and CA as a source of edible natural antioxidants that can BP used by the food industry as an alternative to synthetic antioxidants.

3.4.2 Materials and Methods

Materials

Commercial dried BP and CA were kindly supply from Manatial de la Salut, a registered herbal company in Barcelona, Spain. All reagents and solvents used were analytical grade and obtained from Panreac (Barcelona, Spain) and Sigma Aldrich (England).

Extraction of CA Extract

Dried BP and CA was cleaned and cut and grounded using standard kitchen food processor (Moulinex). Fine grounded plants (2 g) were extracted aqueous solvent (v/v) with either i) 100% ethanol, ii) 75% ethanol, iii) 50% ethanol iv) H_20 v) 50% methanol, in the ratio 1:30 (w/v). All extraction were performed at $4\pm1^{O}C$, light protected for 24 hours and constantly stirred at 1000rpm. Each extraction was carried out triplicates. Then, the extractions were centrifuge to separate the supernatant and the extracts were stored in $-80^{O}C$ for further analysis[15].

Determination of the Total Phenolic Content (TPC)

The Folin-Ciocalteu method was used to determine the total phenolic content as reported by Santas et al. (2008) with some modification[16]. Appropriate dilution of antioxidant extracts were mixed with 80 µL Folin-Ciocalteu reagent and 2% (w/v) Sodium Carbonate. The mixture was finally diluted with miliQ water, shaked and incuBPted in the dark for 1 hour. AbsorBPnce 765 nm was measured using microplate reader (Fluostar Omega, BMG Labtech, Germany) against miliQ water. Gallic acid was used as a standard calibration; the results were expressed as mg of Gallic acid equivalents (GAE) per g dried weight sample (mg GAE/g DW)[16].

Determination of Free Radical Scavenging Activity Assays

b. FRAP assay

The FRAP method used is from Benzie and Strain (1996) with little modifications[4]. The FRAP solution was prepared in a proportion of 10:1:1 of acetate buffer (300 mM) (pH 3.6), 2,4,6-tripyridyl-s-triazine (TPTZ) (10 mM) in HCl (40 mM) and FeCl3 (20 mM), respectively. FRAP solution was incubated at 37oC for 30 minutes before mixing with the appropriate dilution of samples. The absorbance was measured at 593nm using microplate reader. Results were expressed as mg of Trolox equivalents per g dried weight sample (mg TE/g DW).

a. DPPH assay

The effect of extracts on the scavenging of DPPH radicals was determined according to the method adapted from Madhujith and Shahidi (2006) with slight modifications[5]. DPPH reagent (0.1 mM) was dissolved with MeOH and mixed with different concentration of the samples. Dilution from the extract was mixed with DPPH–methanol reagent and the absorBPnce was measured at 517 nm for 90 min. The results were expressed as mg TE/g DW.

c. TEAC assay

The antioxidant capacities of BP and CA were measured by using a modified TEAC assay[6]. The TEAC assay was based on the reduction of the 2,2’-azinobis(3-ethylBPnzothiazoline-6-sulfonic acid)•- radicals cation (ABTS•-)by the antioxidants present in

the samples. Appropriate dilutions were prepared for BP and CA. ABTS•- radical cation (7 mM) was dissolved BPfore adding potassium sulphate (2.45 mM) and allowing the mixture to stand in the dark up to 16 hours. PBS (10 mM) was incuBPted at room temperature for 30 min BPfore used. Then, the mixture of the ABTS•- radical cation was adjusted to an absorBPnce of 0.72 ± 0.2 nm, using microplate reader (Fluostar Omega, BMG Labtech, Germany). The TEAC values for the different concentrations of each compound were interpolated from the Trolox standard curve. The results expressed as mg TE/g DW.

d. ORAC assay

The ORAC value was determined according to Stockham et al (2011) with some amendments[3]. An appropriate concentration of BP and CA extracts were mixed with 13 mM phosphate buffer (incubated at 37°C for 20 minutes) and 80 mM fluorescein respectively. 60 mM APPH radical was added after the initial value of fluorescence was recorded and the fluorescence was monitored for 150 minutes using a microplate reader (Fluostar Omega, BMG Labtech, Germany). The net area under the fluorescein decay curve (AUC) was determined and ORAC values were calculated by comparing the AUC to that of Trolox as a standard. All measured data were expressed as mg of TE/g DW.

Statistical Analysis

Differences between solvent extracts determined by analysis of variance (ANOVA) using the least squares difference method of the General Linear Model in SPSS. Differences were significant at $p < 0.05$.

3.5.4 Results and Discussion

Several principles have to BP considered before making a decision to choose appropriate solvent for plant extraction. Some consideration were according to the purpose of extraction (preparation or analysis), the nature of the assayed components, the physicochemical properties

of the matrix, the availability of reagents and equipment, the cost and safety concerns[18]. The different polarity of solvent employed in the extraction had strong association to the yield and antioxidant activity of the natural plant extract[19].

Table 1: Extraction Yield in Different Solvent Extracts

^{a}**Yield (%)**	**BA**	**CA**
EtOH	4.79 ± 0.18^{b}	1.66 ± 0.01^{b}
75% $EtOH.H_2O$	10.67 ± 0.52^{c}	6.79 ± 0.26^{c}
50% $EtOH.H_2O$	8.69 ± 0.32^{c}	6.22 ± 0.14^{c}
H_2O	7.98 ± 0.11^{c}	4.25 ± 0.17^{c}
50% $MeOH.H_2O$	8.45 ± 0.41^{c}	5.96 ± 0.29^{c}

[a]Extraction yield (%) is calculated according to the method of Zhang and Liu (2007)[17]. Assay was carried out triplicates with less 5% of standard deviation error. [b-c] different letter indicate significant difference ($p<0.05$)

However, the choice of determining the best solvent extraction properties had a lot to consider. The selection must consider each element of assorted structure and composition of the matrix and complex behaviour of each matrix-solvent system which particularly hard to predicted[20]. Thus, in this work, different solvents were assayed for the extraction of BP and CA (water, ethanol, 75% and 50% ethanol aqueous solutions and 50% ethanol aqueous) on the extraction yield, total phenols content and antioxidant activities. Total phenolic content and antioxidant capacity assay were carried out three times in each assay; the values were determined by means of different assay. In all determinations, the percentage of standard deviation was accepted must BP lower than 5%.

Table 1 shows the results obtained of BP and CA extraction yields correspond to their solvent extracts. There are no significantly different on the extraction capacities between aqueous solvents used ($p<0.05$). The highest value of the extract yield obtained in 75% ethanol

aqueous of BP with 10.67 ± 0.52. The extraction yield increase in order: ethanol < water < 50% methanol < 50% ethanol < 75% ethanol. In addition, the increased or decreased of extraction yields were depended on the solvent polarity of extraction[21]. The mixtures of alcohols and water have been more efficient in extracting compounds and better yield than the corresponding mono-component solvent system.

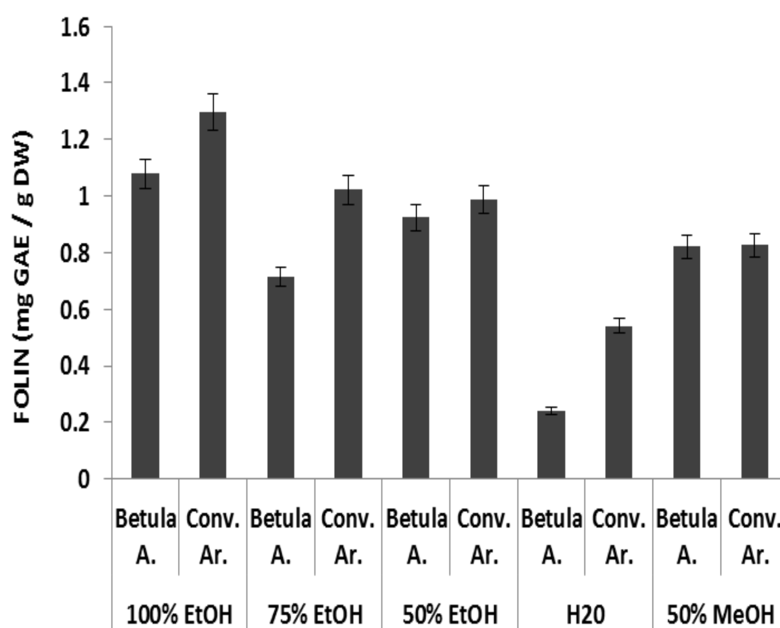


Figure 1: Total phenolic content of BP and CA in different solvent extracts; expressed in mg Gallic acid equivalent (GAE) per g dried weight sample. Assay was carried out triplicates with less 5% of standard deviation error.

The Folin-Cioalteau method had been employed for many years to determine the total phenols in natural products. Although there are some interfering substance in the method such sugars, aromatic amines, sulphur dioxide and ascorbic acid, this method was most popular to determine the total phenol content in the plant sample[22]. Figure 1 shows the total phenolic content for BP and CA in different solvent extract. The extracts with the highest total phenols content were obtained with 100% ethanol in BP and CA, followed by CA in 75% ethanol and BP in 50% ethanol extracts. The lowest value was obtained by water extract. There is almost the same phenol content of CA and BP in 50% methanol extracts with the value of 0.81 ± 0.1. The

significant different was determined by ethanol and water extraction for both plants ($p<0.05$). Many reports claimed the used of binary solvent was the most favourable for extraction of phenolic compounds from plants compared to mono-solvent systems, however, the claimed was contrasted in this study which showed ethanol extract showed the highest phenol content for BP and CA[23,24].

Extraction of active compound in natural plants is potent to protect biological system against damaging effect of natural oxidation process in organism. Thus, potential of antioxidant activity of the compounds extract can BP evaluate by various antioxidant activity assay in this study. In this study, the antioxidant of BP and CA in different solvent extract was evaluate by 4 assays; DPPH, FRAP, TEAC and ORAC. Each antioxidant assay possesses its own unique mechanism to evaluate the antioxidant activity in sample.

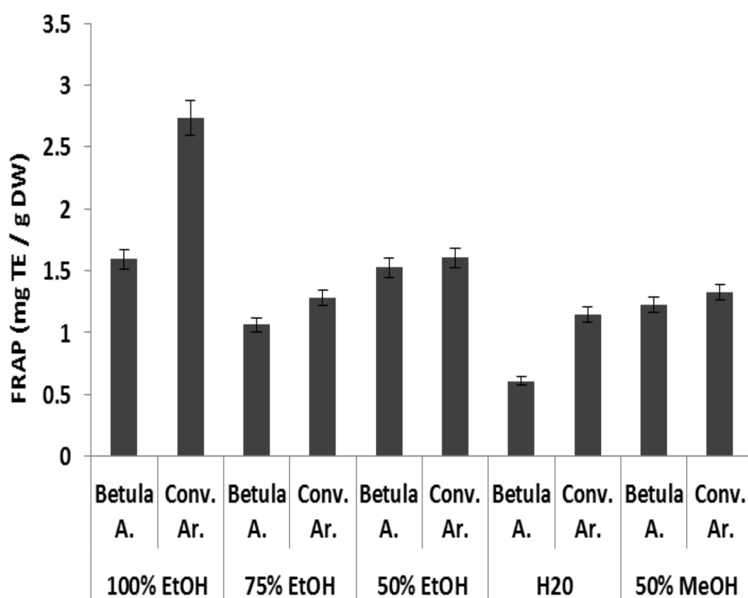


Figure 2: FRAP assay of BP and CA in different solvent extracts; expressed in mg Trolox (TE) per g dried weight sample. Assay was carried out triplicates with less 5% of standard deviation error.

FRAP method was used to present rather quick and simple method measuring antioxidant presents in the BP and CA. The FRAP assay is based on the ability of phenolics to reduce yellow

ferric tripyridyltriazine complex (Fe(III)-TPTZ) to blue ferrous complex (FE(II)-TPTZ) by the action of electron-donating antioxidants[4]. The result of blue colour measured spectrophotometrically at 593 nm is taken as linearly related to the total reducing capacity of electron-donating antioxidants. As shown in Figure 2, ethanol extract showed significant different with all solvent used in this study ($p<0.05$). Absorbance values measured in the extracts varied from 0.61 to 2.66 mg TE / g DW. The aqueous solvent did not exhibit any significant different with water in FRAP method ($p>0.05$). CA extract with pure ethanol showed the highest antioxidant activity in FRAP assay exhibited the significant different from all solvents used. BP extract with water showed the lowest activity however, it was found high antioxidant capacity in both ethanol and ethanol aqueous extraction. In most instances, the ethanol aqueous solvent of BP and CA extracts contained substantial ferric reducing activities compared to the methanol aqueous and water extracts

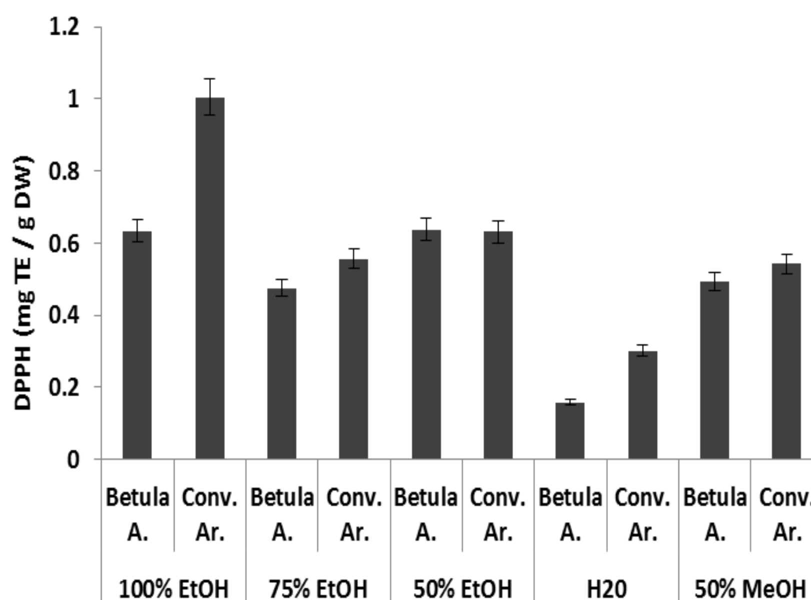


Figure 3: DPPH assay of BP and CA in different solvent extracts; expressed in mg TE per g dried weight sample. Assay was carried out triplicates with less 5% of standard deviation error.

BP and CA extracts scavenging ability were measured by DPPH assay. The DPPH• radical is one of the few stable organic nitrogen radicals and the test is simple and rapid which

probably explains its widespread use in antioxidant screening[22]. In this method, the purple chromogen radical DPPH• is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine25. The loss of DPPH colour after reaction with test compounds was monitored at 517 nm. The discoloration indicates the scavenging potential of the extract, overall, all the extract of BP and CA were able to decolorize DPPH. The activity result was similar to FRAP assay. BP showed lower value compared to CA in water, and methanol aqueous solvents. Nevertheless, CA and BP displayed similar value of antioxidant activity in 50% ethanol extract with 0.63 ± 0.02 mg TE / g DW ($P<0.05$). The DPPH values are measured based on the reducing ability of the plant extract towards DPPH radicals. The extracts obtained with ethanol showed the highest antioxidant activity of CA. Ethanol and ethanol aqueous shows no significant different in BP extract ($p<0.05$).

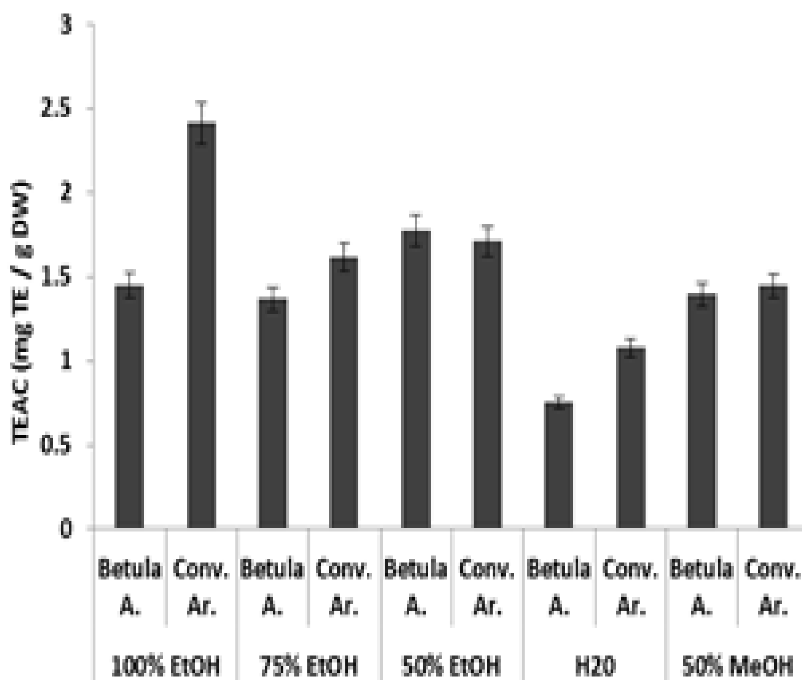


Figure 4: TEAC assay of BP and CA in different solvents extracts; expressed in mg TE per g dried weight sample. Assay was carried out triplicates with less 5% of standard deviation error.

In TEAC assay, CA extracts displayed higher antioxidant value than BP as similar to FRAP and DPPH assays ($p<0.05$). Nevertheless BP extract in 50% ethanol (1.75± 0.05 mg TE / g DW) was the only measurement gives higher antioxidant than CA (1.69 ± 0.13 mg TE / g DW) ($p<0.05$). The assay indicted the plants potency and potential use as a source of antioxidants based on the ability of antioxidants compound to scavenge the long-life radical cation $ABTS^{+}$. The radical anion $ABTS^{\bullet -}$ is generated by $ABTS^{2-}$ oxidation by potassium persulfate. The radical is stable and formed non color diamagnetic compound when reacts by electron transfer with antioxidant[6]. As Figure 4 shows CA ethanol extract and BP extract in 50% ethanol aqueous had the highest capacity to scavenge ABTS radicals and consequently CA in ethanol extract shows the highest antioxidant activity in FRAP and DPPH as well.

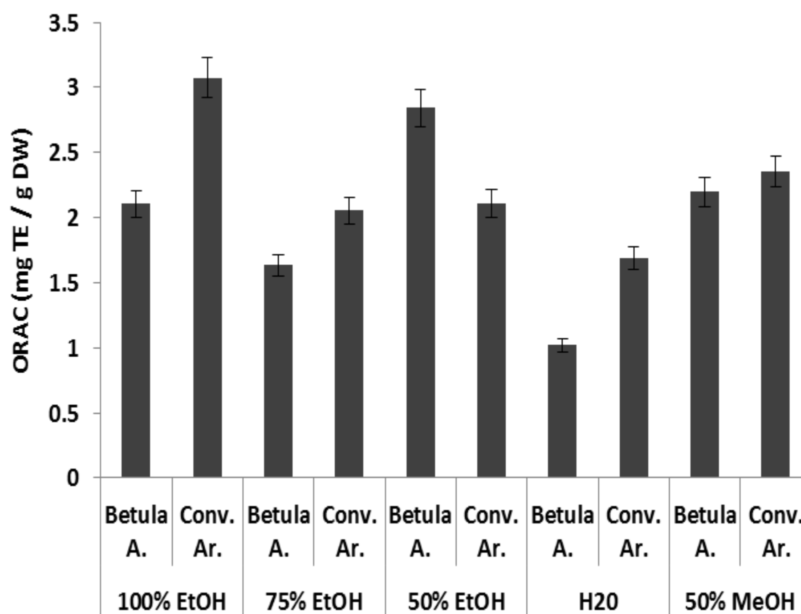


Figure 5: ORAC assay of BP and CA in different solvent extracts; expressed in mg TE per g dried weight sample. Assay was carried out triplicates with less 5% of standard deviation error.

ORAC assay measure the capacity for active compound in plant to scavenge peroxyl radicals generated by spontaneous decomposition of AAPH radicals. The measurement value was estimated in terms of Trolox equivalents similar to TEAC, DPPH and FRAP. This assay

applied to a wide variety of different phytochemicals from edible plants, purified or as an extract or fraction including alkaloids, coumarins, flavonoids, phenylpropanoids, terpenoids, and phenolic acids[14,32,33]. Among the plant extracts assayed here, the values were found to BP in the range between 3.33 ± 0.13 to 1.69 ± 0.06 mg TE / g DW of CA and 2.86 ± 0.11 to 1.03 ± 0.07 mg TE / g DW of BP. CA showed highest antioxidant activity in ethanol and BP extract in 50% ethanol aqueous. 50% methanol extract in CA showed higher activity compared to aqueous ethanol extracts although is not significant ($p<0.05$). The ability of BP and CA extract to scavenge peroxyl radical was showed in ORAC assay and gives the highest value of all antioxidant assays.

Table 2 : Correlation between polyphenol content and antioxidant assay of BP and CA

[a]Assay	[b]R^2
FOLIN vs FRAP	0.7488
FOLIN vs DPPH	0.9032
FOLIN vs TEAC	0.8392
FOLIN vs ORAC	0.7086

[a]Correlation between polyphenol content (FOLIN assay) vs antioxidant capacity assay

[b]Good regression values is accepted from $R^2 \geq 0.7$, and the standard deviation for each assay is less than ±0.5.

The antioxidant assays (FRAP, DPPH, TEAC and ORAC) and FOLIN values of the extracts correlated well, with $R_2 > 0.7$ (Table 2). The FRAP and FOLIN correlation shows the lowest value compare with $R_2 > 0.7488$, meanwhile DPPH reported to have the best correlation with phenolic compound with $R_2 > 0.9032$. Several studies have compared different methods, to evaluate the antioxidant activity of samples, although a general consensus has not yet been established. Some authors find similar values between the methods while others report noticeable differences between them or a dependency on the type of food sample. Nevertheless the good

correlations between phenol content and antioxidant assays confirm that phenols are mainly responsible for the antioxidant activity of extracts. Some study has reported a good correlation between the phenol content of plant extracts and antioxidant activity[16]. Differences in antioxidant activity determined by different methods emphasise the importance of using several methods to assess the parameters in order to obtain accurate data and to improve comparison with other literature.

The different measurement values among antioxidant assay were attributed to the different chemistry principle upon the basis of each method. The DPPH, FRAP and ABTS methods are based on a single electron transfer (SET) reaction. In these methods, antioxidants are oxidised by oxidants, such as a metal (Fe III) or a radical (DPPH or $ABTS^{+}$). As a result, a single electron is transferred from the antioxidant molecule to the oxidant. In contrast, the ORAC assay is based on a hydrogen atom transfer (HAT) reaction. The HAT is transferred after a peroxyl radical ROO has been generated in which this radical extracts a hydrogen atom from the antioxidant compounds. Furthermore, the ORAC assay only measures the activity of chain-breaking antioxidants against peroxyl radicals[22]. Therefore there was relative difference in the measurement of antioxidant assay respectively. The values demonstrated the ability of BP and CA extract either to quench peroxyl radicals or to reduce radicals generated in the assay.

Overall, the influence of the solvent used on extract properties was the same for the total phenols content and antioxidant assays. Extraction of CA in ethanol exhibited the highest total phenolic content and antioxidant activity assay respectively. Ethanol resulted to BP effective in the extraction of flavonoids and their glycosides, catecols and tannins from raw plant materials[26]. Unlike methanol or other strong solvent for extraction such as acetone or chloroform, ethanol is recognised as a GRAS (Generally Recognised as Safe) which positively can BP used for applications in the food industry[21]. However, BP extracts with ethanol and ethanol aqueous showed no significant different on the antioxidant activity values in each antioxidant assay and total phenolic content. ORAC assay showed good scavenging values of BP extract with 50% ethanol.

Determination of the best solvent extract for BP and CA with measurement of TPC and various antioxidant activity assays was one important factor to increase extraction process efficacy. To the best of our knowledge, this study was the first time reported the best solvent

extract of BP and CA for determination of their phenolic compound and antioxidant activity. BP and CA are known in many physiological functions and attracted economic interested to the food industry. These plants were valued for its nutritional, health and sensory attributes however, require more research on their antioxidant activity. BPtulinic acid is a pentacyclic triterpene, one of active compound in BP which displayed range of biological effects, including antiviral, antiparasitic, and antibacterial[9,10,27]. It is also useful as anti-inflammatory activities, and in particular to inhibit growth of cancer cells[8,28]. Methyl syringate compound isolated from BP was antifungal, proved to inhibit for aflatoxin; the most dangerous contaminants occurs in food and feed[29]. CA was believed to have antidiarrhoeal activity[14], cytotoxic effect against a number of tumor cells, immunostimulant effect, anti-bacteria and anti-tumor, anti-angiogenic properties[30]. GC-MS and HPLC results showed the presence of flavonoids, p-hydoxybenzoic acid, syringic acid, vanillin, benzoic acid and ferulic acid were responsible for the antioxidant activity[14]. Moreover, the preliminary literature suggested that BP and CA were having their unique compounds for the benefit of neutraceutical, pharmaceutical and medicinal used. Thus, this research was extended to evaluate the antioxidant capacity of these plants as a primary study for alternative source of natural antioxidant for food commodities.

3.5.4 Conclusions

In summary, this study has revealed that a range of values for total antioxidant capacities and phenolic contents exist among the *Betula Pendula Roth.* and *Convolvulus Arvensis*. Varies of measurement related to different solvent concentration in the extraction and which antioxidant activity assay had been performed. Overall, CA displayed the highest antioxidant activity in ethanol extract and BP shows varies measurement in antioxidant assays and total phenolic content respectively. The good correction between antioxidant assays and total phenolic content were determined for BP and CA extracts. Thus, this study was performed useful to evaluate the antioxidant capacity in various assays that supports the initial study for its potential sources of potent natural antioxidant

References

1. Aruoma OI. Free radicals, oxidative stress and antioxidants in human health and disease. *J. Am. Oil Chem. Soc.* 1998;75:199-212.

2. Aruoma OI. Antioxidant actions of plant foods, use of oxidative DNA damage as a tool for studying antioxidant efficacy. *Free Radical Res*. 1999;30:419-427.

3. Stockham K. Paimin R. Orbell JD, Adorno P, Buddhadasa S. Modes of handling Oxygen Radical Absorbance Capacity (ORAC) data and reporting values in product labelling *J.Food Compos.Anal*, 2011;24:686-691.

4. Benzie I and Strain J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal Biochem.* 1996;239:70-76.

5. Madhujith T and Shahidi F. Optimization of the extraction of antioxidative constituents of six barley cultivars and their antioxidant properties. *J. Agric. Food. Chem.* 2006;54:8048-8057.

6. Miller NJ, Sampson L, Candeias LP, Bramley PM, RiceEvans CA. Antioxidant activities of carotenes and xanthophylls. *FEBS Letters*. 1998;384:240-242.

7. García-Plazaola JI, Hernández A, Becerril JM. Antioxidant and Pigment Composition during Autumnal Leaf Senescence in Woody Deciduous Species Differing in their Ecological Traits. *Plant boil*. 2003;5:557-566.

8. Einzhammer DA and Xu ZQ. Betulinic acid: a promising anticancer candidate. *IDrugs*. 2004;4:359–373.

9. Thurnher D, Turhani D, Pelzmann M, Wannemacher B, Knerer B, Formanek M, Wacheck V, Selzer E. Betulinic acid: a new cytotoxic compound against malignant head and neck cancer cells. *Head Neck.* 2003;25:732–740.

10. Eder-Czembirek C, Czembirek C, Erovic BM, Selzer E, Turhani D, Vormittag L, Thurnher D. Combination of betulinic acid with cisplatin-different cytotoxic effects in two head and neck cancer cell lines. *Oncol Rep.* 2005;14:667–671.

11. Ehrhardt H, Fulda S, Fuhrer M, Debatin KM, Jeremias I. Betulinic acid-induced apoptosis in leukemia cells. *J Leukemia.* 2004;8:1406–1412.

12. Raghuvar DV, Gopal A, Nakar A, Badrinath Y, Mishra KP, Joshi DS. Betulinic acid induces apoptosis in human chronic myelogenous leukemia (CML) cell line K-562 without altering the levels of Bcr-Abl. *Toxicol Lett.* 2005;15:343–351.

13. Roberto C. Medicinal Plants. London:The Macdonald & Co. Puplisher Ltd and Sydney, 1982, ch. 95.

14. Al-Bowait IF and Albokhadaim AM. Immunostimulant Effect of Bindweed (*Convolvulus Arvensis*)Extract in Rabbit. *Research Journal of Pharmacology*. 2010;4:51-54.

15. Sadeghi-aliabadi H, Ghasemi N, Kohi M. Cytotoxic effect of *Convolvulus arvensis* extracts on human cancerous cell line. *Research in Pharmaceutical Sciences*. 2008;3:31-34.

16. Santas J, Carbo R, Gordon MH, Almajano MP. Comparison of the antioxidant activity of two Spanish onion varieties. *Food Chem*. 2008;107:1210-1216.

17. Zhang S, Bi H, Liu C. Extraction of bio-active components from Rhodiola sachalinensis under ultrahigh hydrostatic pressure. *Sep Purif Technol*. 2007;57:275−280.

18. Yu L, Haley S, Perret J, Harris M. Antioxidant properties of hard winter wheat extracts. *Food Chem*. 2002;78:457–461.

19. Moure A, Cruz JM, Franco D, Dominguez H, Sineiro J, Dominguez H, Núnez MJ, Parajó JC. Natural antioxidants from residual sources. *Food Chem*. 2001;72:145–171.

20. M. Al-Farsi, C. Y. Lee, "Optimization of phenolic and dietary fibre extraction from date seeds," Food Chemistry, vol.108, pp.977–985, 2008.

21. Fernández-Agullóa A, Pereira E, Freire MS, Valentão P, Andrade PB, González-Álvareza J, Pereira JA. Influence of solvent on the antioxidant and antimicrobial properties of walnut (*Juglans regia L.*) green husk extracts. *Ind Crop Prod.* 2013;42:126– 132.

22. Prior R, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J Agr Food Chem*. 2005;53:4290–4302.

23. Spigno G, Tramelli L, De Faveri DM. Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. *J. Food Eng*. 2007;81:200–208.

24. Chew KK, Khoo MZ, Ng SY, Thoo YY, Wan Aida WM, Ho CW, "Effect of ethanol concentration, extraction time and extraction temperature on the recovery of phenolic compounds and antioxidant capacity of Orthosiphon stamineus extracts," *International Food Research Journal*. 2011;18:1427–1435.

25. Magalhaes LM, Segundo MA, Reis S, Lima JLFC. Methodological aspects about in vitro evaluation of antioxidant properties. *Analytica Chimica ACTA.* 2008;613:1-19.

26. Spigno G, Tramelli L, De Faveri DM. Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. J. Food Eng. 2007;81:200–208.

27. Jussi-Pekka R, Remes S, Heinonen M, Hopia A, Kahkonen M, Kujala T, Pihlaja K, Vuorela H, Vuorela P. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Int J Food Microbiol*. 2000;56:3–12.

28. Rzeski W, Stepulak A, Szymański M, Sifringer M, Kaczor J, Wejksza K, Zdzisińska B, Kandefer-Szerszeń M. Betulinic acid decreases expression of bcl-2 and cyclin D1,

inhibits proliferation, migration and induces apoptosis in cancer cells," *N-S Arch Pharmacol.* 2006;374:11–20.

29. Jermnak U, Yoshinari T, Sugiyama Y, Tsuyuki R, Nagasawa H, Sakuda S. Isolation of methyl syringate as a specific aflatoxin production inhibitor from the essential oil of Betula alba and aflatoxin production inhibitory activities of its related compounds. *Int J Food Microbiol*. 2012;153:339–344.

30. Attia HA, Samar M, Mouneir M. Antidiarrhoeal activity of some Egyptian medicinal plant extracts," *J Ethnopharmacol.*2004;92:303–309.

3.6. A Study of the Properties of Bearberry Leaf Extract as Natural Antioxidant in Model Foods

Abstract

Common Bearberry (*Arctostaphylos uva-ursi L. Sprengel*) is a ubiquitous procumbent evergreen shrub located throughout North America, Asia and Europe. The fruits are almost tasteless but the plant contains a high concentra-tion of active ingredients. The antioxidant activity of Bearberry leaf extract in the 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical cation assay was 90.42 mmol Trolox equivalents/g DW. The scavenging ability of the methanol extract of Bearberry against methoxy radicals generated in the Fenton reaction was measured by Electron Paramagnetic Resonance. Lipid oxidation was retarded in an oil-water emulsion by add-ing 1 g/kg lyophilised Bearberry leaf extract. Also, 1 g/kg of lyophiliesd Bearberry leaf extract incorporated into a gelatin based film displayed high antioxidant activity to retard degradation of lipid in muscle foods. The present results indicated the potential of bearberry leaf extract for use as a natural food antioxidant.

Keywords:

Bearberry leaves, Scavenging activity, Lipid oxidation, Active packaging film, Antioxidant activities

3.6.1 Introduction

Lipid oxidation in food causes serious problems that lead to short shelf lives and loss of nutritional quality[1]. Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ) have been used as antioxidants in many foods products[2], but consumers have become concerned about possible toxicological effects and often prefer natural antioxidants for foods consumed as part of a healthy diet. Thus, many investigations have focused on identification of novel antioxidants and these have been tested in model foods such as emulsions and incorporated into packaging films.

Natural antioxidants contain a high concentration of phenolic compounds and normally occur in fruits, vegetables and herbs[3,4]. Bearberry (*Arctostaphylos uva-ursi L. Sprengel*) is a ubiquitous procumbent evergreen shrub located throughout North America, Asia, and Europe. The fruits are almost tasteless despite containing a high concentration of active ingredients in many commercial products[5]. The antioxidant potential of bearberry leaves (BL) has been studied by numerous chemical assays including reducing power assay, DPPH scavenging activity, a liposome model, scavenging hydroxyl radicals (HO) and a linoleic acid model system[6,7]. The main constituents of BL are the glycosides arbutin (5–15%), and methylarbutin (up to 4%) and small quantities of the free aglycones. Other constituents include ursolic acid, tannic acid, gallic acid, p-coumaric acid, syringic acid, galloylarbutin, gallotannins, and flavonoids, notably glycosides of quercetin, kaempferol, and myricetin according to Barl et al. (2007)[8]. The traces of polyphenols in BL have been made them promising candidates as potential protectors against lipid oxidation and biological ageing of tissues.

Additionally, different phenolic compounds may act as antioxidants with varying efficiency in different food a system which depends on their polarity and molecular characteristics. Several studies of the antioxidant activity of BL at several concentrations have been conducted in a meat model[9,10] and these have successfully demonstrated the potential of bearberry to inhibit the degradation of lipids in pork. However, the effect of BL extract in oil-water emulsions has not been investigated. There is also limited information on the utilisation of the plant extracts incorporated into a film as active packaging. Therefore, this investigation

aimed to: (a) investigate the potential antioxidant properties of BL extract using the TEAC assay and EPR scavenging activity (b) demonstrate the ability of BL lyophilise to inhibit lipid deterioration directly in oil water emulsions (c) study the effectiveness of gelatin based film treated with lyophilised BL in retarding lipid oxidation in meat patties.

3.6.2 Materials and Methods

Materials

Commercial dried BL was kindly supplied by Pàmies Hortícoles (Balaguer, Spain), a registered herbal company. All reagents and solvents used were analytical grade and obtained from Panreac (Barcelona, Spain) and Sigma Aldrich (Gillingham, England)

Extraction of Bearberry leaves.

Dried BL were finely ground using a standard kitchen food processor. Ground BL was extracted with 50:50 (v/v) ethanol:water always in the ratio 1:20 (w/v). The extractions were performed at 4±1°C for 24 h, in the dark with constant stirring. The solutions of BL extract were recovered by filtration using Whatman Filter paper, 0.45 µm. Part of the supernatant was taken for subsequent use to determine the antiradical capacity. The volume of the remaining supernatant was measured and the excess ethanol was removed under vacuum using a rotary evaporator (BUCHI RE111, Switzerland) and kept frozen at -80 °C for 24 hours. All extracts were dried in a freeze dryer (Unicryo MC2L -60°C, Germany) under vacuum at -60 °C for 3 days to remove moisture. Finally, lyophilised BL were weighed to determine the soluble concentration (g/l) as described by Zhang et al. (2007)[11].

Determination of the Total Phenolic Content (TPC)

The Folin-Ciocalteu method was used to determine the total phenolic content as described by Santas et al.(2008)[12].

Determination of antioxidant activity using TEAC Assay

The antioxidant capacity of BL was measured by a modified TEAC assay as described by Skowyra et al. (2013)[13], which was based on the method of Miller et al. (1996)[14].

Electron Paramagnetic Resonance (EPR) Spectroscopy Radical Scavenging assay

EPR radical scavenging activity was measured following the method of Azman et al. (2014)[15]. BL were extracted with MeOH in a ratio of 1:10 (w/v) and the soluble concentration of BL was determined as described in the procedure above. A spin-trapping reaction mixture consisted of 100 μL of DMPO (35 mM); 50 μL of H_2O_2 (10 mM); 50 μL BL extract solution at different concentrations or 50 μL of ferulic acid used as reference (0−20 g/L) or 50 μL of pure MeOH used as a control; and, finally, 50 μL of $FeSO_4$ (2 mM), added in this order. The final solutions (125 μL) were tranferred to a narrow (inside diameter = 2 mm) quartz tube and introduced into the cavity of the EPR spectrometer. The spectrum was recorded 10 min after the addition of the $FeSO_4$ solution, when the radical adduct signal was greatest. X-band EPR spectra were recorded with a Bruker EMX-Plus 10/12 spectrometer under the following conditions: microwave frequency, 9.8762 GHz; microwave power, 30.27 mW; center field, 3522.7 G; sweep width, 100 G; receiver gain, 5.02 × 104; modulation frequency, 100 kHz; modulation amplitude, 1.86 G; time constant, 40.96 ms; conversion time, 203.0 ms.

Determination of Antioxidant Activity in o/w Emulsion

a. Removal of Tocopherols from Sunflower Oil

Alumina was placed in an oven at 200 °C for 24 h, and then removed and allowed to cool in a desiccator until it reached room temperature. Sunflower oil was passed twice through the alumina in a column to remove the tocopherols as described by Yoshida et al.(1993)[16]. Finally, the filtered oil was stored at −80 °C until use.

b. Preparation of Emulsion

Oil in water emulsion emulsion was prepared by a method adapted from Azman et al.(2014)[17]. The final samples were prepared either (i) control (no addition); (ii) 0.2 g/kg BHA; (iii) 1 g/kg lyophilised BL. The emulsion for each sample was prepared in quadruplicate, obtaining a total of 12 samples and stored in the dark and allowed to oxidize at 37 °C. The pH of the samples was measured four times for each sample (pH meter GLP21, Crison Instruments, Barcelona, Spain) as a parameter to investigate its correlation with PV.

c. Determination of Peroxide Value (PV)

The primary oxidation products were measured using peroxide value (PV) according to the thiocyanate method of the Association of Official Analytical Chemists (AOAC) 8195[18]. Ferrous chloride solution was prepared in hydrochloric acid (1 M) with the addition of iron chloride (II) (2 mM, final concentration). Ammonium thiocyanate solution was prepared in water (2 mM, final concentration). The assay was performed with a drop of emulsion in the range from 0.007 to 0.01 g, diluted with ethanol. From this solution the required amount of sample, varying according to the degree of oxidation, was taken in a cuvette and ethanol was added. Ferrous chloride and ammonium thiocyanate solutions were added, each in a proportion of 1.875% (v/v), final concentration. The absorbance was measured spectrophotometrically at $\lambda = 500$ nm. The results are expressed as meq hydroperoxides/kg of emulsion.

d. Preparation of gelatine based film with antioxidant coating

The fabrication of gelatin based film with antioxidant coating was adapted and characterized by the method of Bodini et al.(2013)[19]. During the cooling of the filmogenic solution after the solubilization of sorbitol, 1 g /kg of BL extract / gelatin was added. Fat and joint tissues were trimmed off lean meat (2000g) and the meat was minced through 8mm industrial plates. Then, the meat was moulded to a thickness of 1.5 cm. For each slice, films (5×5 cm2) were placed on both sides either with control film (no addition of antioxidant) and BF film (1 g/kg lyophilised BF). Control sample were prepared in the same manner except that the slices

were not covered with any film. Subsequently, the samples were packed in polypropylene trays prior to storage at 4 °C for 12 days.

e. Thiobarbituric acid reacting substances (TBARS)

TBARS measurement was used to measure the extent of lipid oxidation during the storage period as de-scribed by Grau et al. (2000)[20]. Sample (1 g) was weighed in a tube and mixed with 3 ml/L aqueous EDTA. Then, the sample was immediately mixed with 5 ml of thiobarbituric acid reagent using an Ultra-Turrax (IKA, Germany); at 32000 rpm speed, for 2 min. All procedures were carried out in the dark and all samples were kept in ice. The mixture was incubated at 97±1oC in hot water for 10 minutes and shaken for 1 minute during the process to form a homogeneous mixture. The liquid sample was recovered by filtration (Whatman Filter paper, 0.45 µm) after the sample was cooled for 10 minutes. The absorbance value for each sample was measured at 531 nm using a spectrophotometer. The TBARS value was calculated from a MDA standard curve prepared with 1,1,3,3 tetraethoxypropane and analysed by linear regression. All results were reported in mg malonaldehyde (MDA)/kg sample.

Statistical Analysis

Differences between samples at each day of storage were determined by analysis of variance (ANOVA) using the least squares difference method of the General Linear Model in SPSS. Differences were identified as significant at $p < 0.05$.

3.6.4 Results and Discussion

Extraction yield, total phenolic content (TPC) and antioxidant activity

The extraction yield, total polyphenols (TPC) and antioxidant activity in extracts of Bearberry leaves ob-tained with 50:50 v/v ethanol:water are shown in Table 1. On average, 1.6 ±

0.01 g of extract pulp was recovered from 5g of Bearberry extract after 3 days freeze drying ($p>0.05$).

Table 1: Extraction yield, polyphenol content and antioxidant activity of Bearberry leaves extracts.

Activity Bearberry extract	Extraction solvent 50:50 (v/v) EtOH:H_2O
[a] Extraction yield (%)	32.1 ± 0.03%
[b] Total phenolic content (mg GAE g^-1 DW)	102.11 ± 7.12
[c] TEAC (mmol of TE g^-1 DW)	90.42 ± 1.83

* Results are expressed as mean ± standard deviation (n = 3).

Pegg et al.(2005) reported that the total phenolic content of BL extract was 312 mg/g DW for 95% (v/v) ethanol extraction[6], much higher that what we reported for 50% (v/v) ethanol extract. However, the total phenolic content value obtained from the BL water infusion was very low with 160.78 ± 2.84 g/kg sample[21]. The mixtures of alcohol and water have been more efficient in extracting phenolic compounds and gave a better yield than water since some phenolic constituents do not dissolve in water. Meanwhile, the antioxidant activity of BL assessed using the TEAC method was 90.42 mmol of TE/gDW. A recent study reported the antioxidant activity of BL in the TEAC assay was 3.19 ± 0.01 molTE/ kg sample following extraction by an infusion method which is much lower than our value[21]. The antioxidant activity of bearberry leaf achieved in the TEAC assay indicates the potency of the extract to scavenge the radical cation $ABTS^{\bullet +}$ generated in the assay. The use of several methods allows a more general assessment of the antioxidant properties of the plant. The variations of data were influenced by the sample preparation, type of extraction (solvent, temperature, etc.), selection of end-points and

method of expression of the results. Several studies have determined the antioxidant activity of BL extract using in-vitro analysis. A few studies reported the bearberry extract scavenging ability using the DPPH radical and the ability of the extract to reduce ferric(III) ions to ferrous(II) ions using the FRAP method[9,21]. The polyphenol constituents in the extract contribute most of the antioxidant activity. The infusion of BL showed an abundance of phenolic acid components at trace concentrations including catechin and its derivatives, epigallocatechingallate, epigallocatechin and epicatechin. These catechins have a strong antioxidant capacity mainly linked to their radical scavenging activity[15].

EPR scavenging radical assay

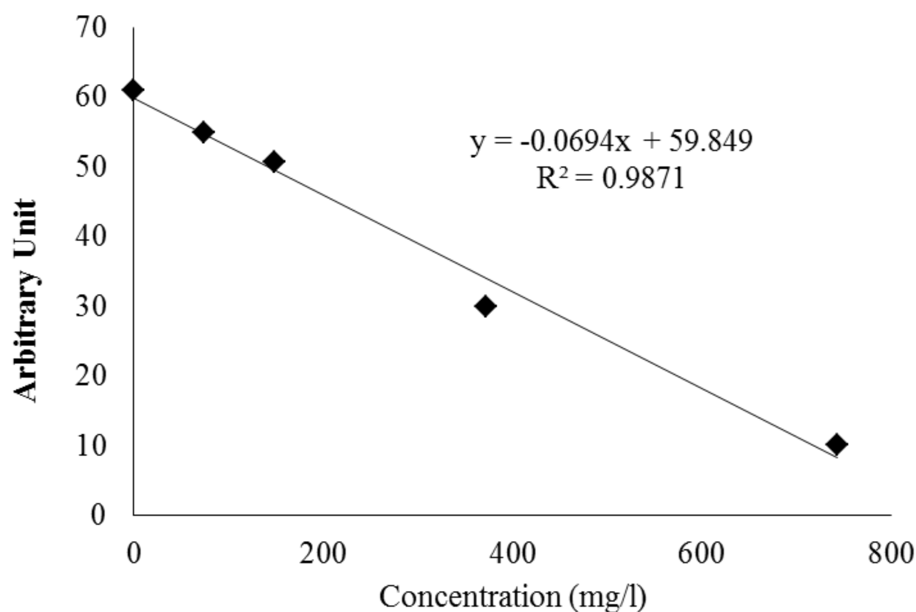


Figure 1: Variation in the area of the electron paramagnetic resonance (EPR) spectra of the radical adduct DMPO-OCH_3 generated from a solution of H_2O_2 [2 mM] and $FeSO_4$ [0.04 mM] with DMPO [14 mM] as spin trap in MeOH as solvent. The decreases of the EPR signal with the increase of concentration of the BL methanol extracts. The EPR signal decreases with the higher antioxidant activity.

In the present study, methanolic extracts from BL were examined by EPR spectroscopy for their capacity to act as radical scavenger towards the methoxy radical ($CH3O^\bullet$) generated by the Fenton reaction. This method has been used for the first time to evaluate the scavenging ability of plant extracts for the free methoxy radical ($CH3O^\bullet$). Figure 1 shows the decrease in the EPR signal with increasing concentration of BL extract. The free radical scav-enging activity of the extracts against methoxy ($CH3O^\bullet$) radical was investigated by a competitive method in the presence of DMPO as spin trap and recorded by the spectrum generated by EPR spectroscopy. The $CH3O^\bullet$ radical generated by to the Fenton procedure has a relatively short half-life that means it must be identified by EPR as the stable nitroxide adduct with DMPO, DMPO-OCH_3 (hyperfine splitting constants, aN = 13.9 G and aH = 8.3 G). This stable DMPO-OCH_3 compound can be quantified by the double integration value of the signal from EPR. The extract containing antioxidant at different concentrations may compete with the spin trap DMPO in the scavenging of methoxy radicals. Thus, the effect decreases the amount of radical adducts and, accordingly, decreases the intensity of the EPR signal. The best fit with intensity of EPR signal was shown as a linear function (Figure 1) that, if concentration values, x, are in g/L, corresponds to equation (1):

The graph indicates the exponential value of the signal of the spectrum decreased as the amount of bearberry extract increased. Azman et al. (2014) demonstrated the scavenging ability of catechins with methoxy radical using this assay[15]. These catechins was also found in the bearberry extract by Valjkovic et al. (2013) and and these compounds contribute to the ability to scavenge methoxy radical in this assay[21]. Furthermore, BL scavenging ability has been previously reported by Amarowicz et al. (2004) by free hydroxyl free radical ($HO^\bullet$) measured in EPR[9].

Antioxidant Effect in Stored o/w Emulsion

Methods have been developed to understand the effect of natural antioxidants in model foods such as emulsions and active film packaging. Adding natural antioxidants to food not only delays the oxidation process but also enhances the nutritional quality of the food through direct ingestion. In previous work, the effect of Bearberry leaf extract in oil-water emulsion has not

been described. A model emulsion has been used to assess the deteriora-tion of lipid at two stages of oxidation, the primary oxidation products (Peroxide Value) and secondary oxidation products (TBARS). In addition the change in pH was monitored, since pH tends to fall during oxidation.

a. Evolution of Peroxide Value (PV)

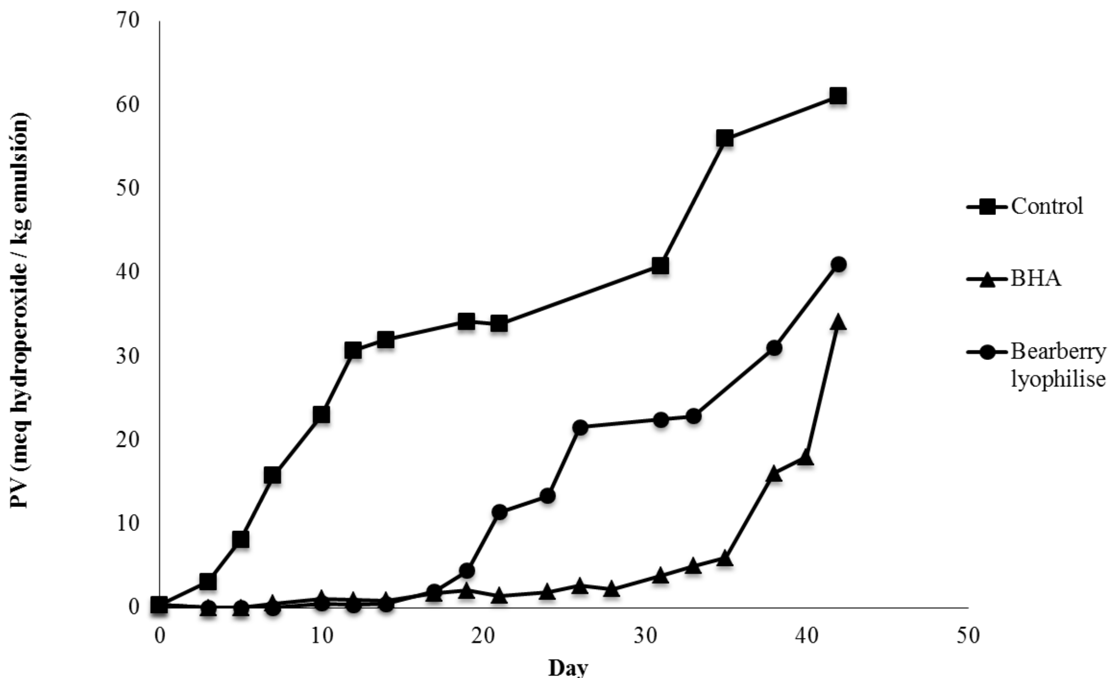


Figure 2: Change of peroxide value over time stored at 37 °C. (each value is expressed as mean (n = 3)).

The development of primary oxidation products was monitored by evaluation of hydroperoxide formation (PV) during storage with results as shown in Figure 2. Primary degradation of lipids measured by PV occurs due to the reaction between oxygen and unsaturated fatty acids that form hydroperoxides. The induction time is defined as the time for samples to reach 10 meq hydroperoxides/kg of emulsion. This value can be used as a measure of the stability of emulsions. The limits of oxidation products in fat products (animal, plant and anhydrous) including margarine and fat preparations were set at <10 meq hydroperoxides/kg as a guarantee of the product quality[17]. When the peroxide value of the sample is greater than 10 meq

hydroperoxide/kg, the sample is in a highly oxidised state and starts to become rancid. The PV value of the control emulsion increased rapidly, reaching more than 10 meq hydroperoxide/kg after only 6 days ($P<0.05$). The sample containing BL 1 g/kg reached the end of the induction time after 20 days while the BHT samples reached this state after 36 days storage. Several studies have investigated the effects of adding natural antioxidants to delay the lipid deterioration in food model emulsions. Skowyra et al.(2013)[13] found that emulsion containing 48 µg/mL of Tara extract took 13 days to reach more than 10 meq hydroperox-ide/kg and emulsion containing green tea extract required 8 days to reach the end of the induction time as reported by Roedig-Penman and Gordon (1997)[22]. Emulsion containing 100 mg/L of rosemary and thyme extract displayed low PV which remained below 10 for 25 days storages[23].

b. Evolution of pH

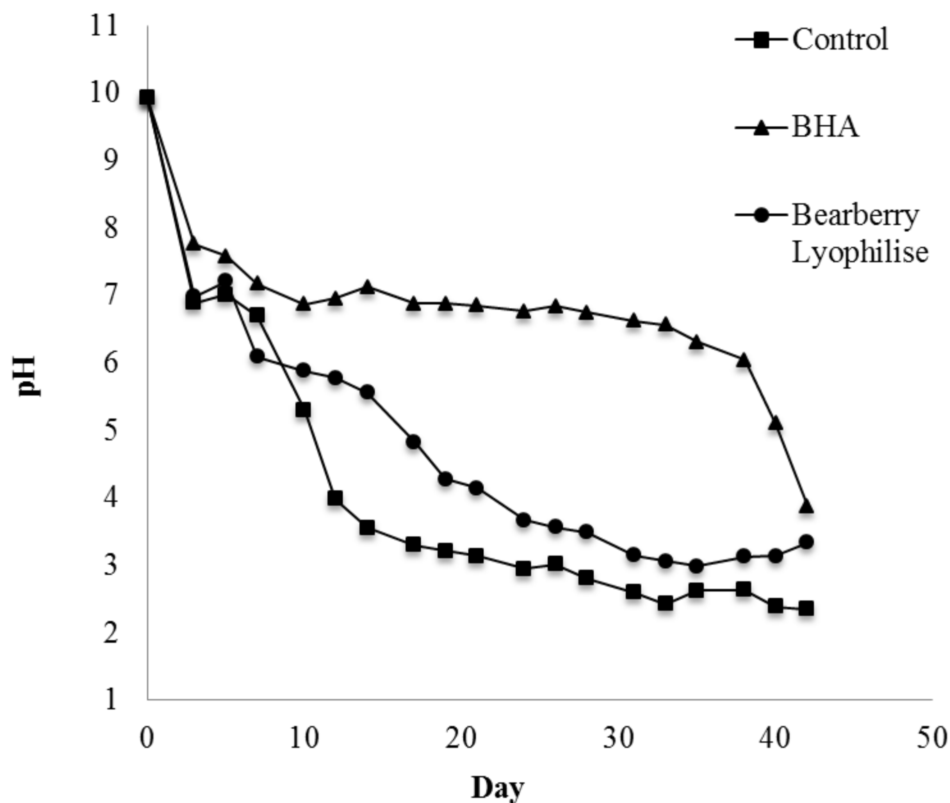


Figure 3: Change of pH over time stored at 37 °C (each value is expressed as mean (n = 3)).

Primary oxidation occurs rapidly in the fat phase of the product due to the formation of hydroperoxides which are highly unstable and break down easily. This process results in the formation of ketones, epoxides or organic acids which are acidic and lead to changes in the pH[13]. Overall, the pH value dropped over time as shown in Figure 3, and this change is inversely to the increase of PV. By comparison, emulsion containing BHA showed a significant difference in pH from the values for BL and the control sample ($p<0.05$). BL samples remained higher than pH 6 for 10 days storage and the pH declined gradually until 40 days.

A number of authors have suggested a positive effect of pH on oxidation rate which is influenced by natural antioxidants[13,23]. Pehlivan et al. (2008) reported that edible vegetable oils contain heavy metals such as iron up to 0.2 mg/kg oil. The levels of some metal compounds if high enough will promote oxidation which affects the pH. Furthermore, the redox state of metals and the activity, solubility, stability, and chelation capacity of antioxidants are among the parameters that affect the rate of change of pH in oil emulsions[26].

c. TBARS in active film packaging with Bearberry coating

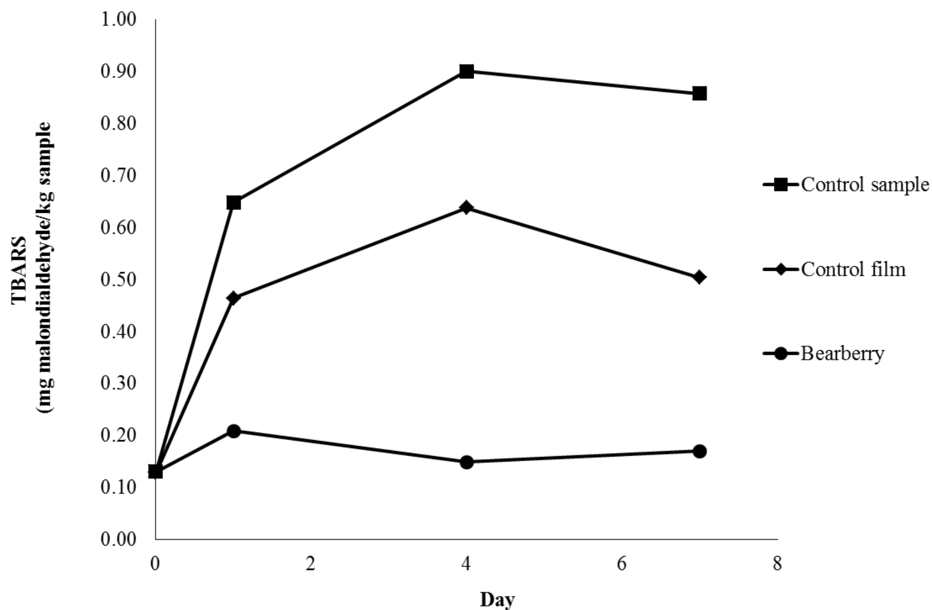


Figure 4: Changes in TBARS values (mg malodialdehyde/kg sample) of control and sample containing and BL extract during 7 days storages at $4 \pm 1^{O}C$ without light. (Each sample was measured in triplicate and the average standard deviation for each sample was less than 5%).

The TBARS index (Figure 4) revealed that secondary oxidation of the control beef patties increased pro-gressively as storage advanced. Meat patties without any film on the patties (control sample) experienced the highest TBARS values and the values were significantly different to those of all other samples. Coating with gelatin based film enriched with BL extract lowered the oxidation rate significantly throughout storage ($p<0.05$). At the end of the storage time, patties stored under the gelatin film with BL contained only 0.17 mg malondialdehyde / kg sample compared to the control TBARS value of 0.86 mg malondialdehyde / kg sample. There are very few reports dealing with the effects of edible gelatin based films containing natural plant extracts. Published studies of gelatin based films have focused on the physical, chemical and mechanical properties of the film[19,27]. However, there are only a few studies which report the antioxidant effects of gelatin-film treated with various natural antioxidants. Several literature reports have recently proposed that gelatin should be combined with plant extracts containing phenolic compounds to improve its physical properties as well as to introduce active properties causing delays in the oxidation of foods within the food packaging[28,29]. This study has reported the preliminary results of the effects of bearberry extract incorporated in gelatin film which successfully delayed oxidation in a muscle food.

3.6.4 Conclusion

In conclusion, this study clearly showed the positive effect of BL extract due to its antioxidant activity, and scavenging ability, which delayed lipid oxidation in an emulsion and when used as an active componentb in gelatin film packaging. BL extract contained a high concentration of phenolic compounds and good antioxidant activity when assessed by the total phenolic content and TEAC assay respectively. The methanol extract showed scavenging ability against methoxy radicals generated by the Fenton Reaction when assessed by EPR. Lyophilised BL (0.1% w/w) was applied as an antioxidant in an emulsion food model and significantly inhibited lipid oxidation during 20 days storage. A preliminary study of the effect of gelatin based film coated with BL extract showed that it significantly delayed degradation of lipids in

meat patties ($p<0.05$). Therefore, this study confirmed that Bearberry leaves can be used as a source of antioxidants with potential for use by the food industry.

References

1. Reische DW, Lillard DA, Eitenmiller RR. InC. Akoh & DM (ed) Food Lipid Chemistry, Nutrition and Biotechnology. 2002. 2nd edn, Dekker, New York.

2. Jayathilakan K, Sharma GK, Radhakrishna K, Bawa AS. Antioxidant potential of synthetic and natural antioxidants and its effect on warmed-over-flavour in different species of meat. *Food Chem.* 2007;105:908-916.

3. Roby MHH, Sarhan MA, Selim KA-H, Khalel KI. Evaluation of antioxidant activity, total phenols and phenolic compounds in thyme (*Thymus vulgaris L.*), sage (*Salvia officinalis L.*), and marjoram (*Origanum majorana L.*) extracts. *Ind Crops Prod.* 2007;43:827-831.

4. Argoti JC, Salido S, Linares-Palomino PJ, Ramírez B, Insuasty B, Altarejos J. Antioxidant activity and free radical-scavenging capacity of a selection of wild-growing Colombian plants. *J Sci Food Agric* 2011;91:2399-2406.

5. Naczk M, Pegg RB, Amarowicz R. Protein-precipitating capacity of bearberry-leaf (Arctostaphylos uva-ursi L. Sprengel) polyphenolics. *Food Chem*. 2011;124:1507-1513.

6. Pegg RB, Amarowicz R, Naczk M. Antioxidant Activity of Polyphenolics from Bearberry Leaf (*Arctostphylos Uva-Ursi L. Sprengel*) Extract in Meat Systems. *J. Am. Chem. Soc*. 2009; 909:67-82.

7. Pegg RB, Amarowicz R., Naczk M, Shahidi F. PHOTOCHEM_ for Determination of Antioxidant Capacity of Plant Extract. *J. Am. Chem. Soc*. 2009;956:140-158.

8. Barl B, Loewen D, Svendsen E. 1996. Saskatchewan Herb Database. Canada

9. Amarowicz R, Pegg RB, Rahimi-Moghaddam P, Barl B, Weil JA. Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. *Food Chem.* 2004;84:551-562.

10. Carpenter R, O'Grady MN, O'Callaghan YC, O'Brien NM, Kerry JP. Evaluation of the antioxidant potential of grape seed and bearberry extracts in raw and cooked pork. *Meat Sci.* 2007;76:604-610.

11. Zhang S, Bi H, Liu C. Extraction of bio-active components from Rhodiola sachalinensis under ultrahigh hydrostatic pressure. *Sep Purif Technol*. 2007;57:277-282.

12. Santas J, Carbo R, Gordon M, Almajano M. Comparison of the antioxidant activity of two Spanish onion varieties. *Food Chem.* 2008;107:1210-1216.

13. Skowyra M, Falguera V, Gallego G, Peiró S, Almajano MP. Antioxidant properties of aqueous and ethanolic extracts of tara (Caesalpinia spinosa) pods in vitro and in model food emulsions. *J Sci Food Agric.* 2014; 94:911-8.

14. Miller NJ, Sampson J, Candeias LP, Bramley PM, Rice-Evans CA. Antioxidant activities of carotenes and xanthophylls. *FEBS Lett.* 1996;384:240-242.

15. Azman NAM, Peiró S, Fajarí L, Julià L, Almajano MP. Radical scavenging of white tea and its flavonoid constituents by electron paramagnetic resonance (EPR) spectroscopy. *J Agric Food Chem.* 2014;62:5743-5748.

16. Yoshida H, Kajimoto G, Emura S. Antioxidant effects of d-tocopherols at different concentrations in oils during microwave heating. *J Am Oil Chem Soc.* 1993;70:989-995.

17. Azman N, Segovia F, Martínez-Farré X, Gil E, Almajano M. Screening of Antioxidant Activity of Gentian Lutea Root and Its Application in Oil-in-Water Emulsions. *Antioxidants.* 2014;3:455-471.

18. Peroxide Value Acetic Acid-Chloroform Method. In: AOCS Official Method Cd. 1997;8-53.

19. Bodini RB, Sobral PJ, Favaro-Trindade CS, Carvalho R. Properties of gelatin-based films with added ethanol–propolis extract. *LWT - Food Sci Technol* 2013;51:104-110.

20. Grau A, Guardiola F, Boatella J, Barroeta A, Codony R. Measurement of 2-thiobarbituric acid values in dark chicken meat through derivative spectrophotometry: influence of various parameters. *J Agric Food Chem* 2000;48:1155-1159.

21. Veljkovi JN, Pavlovi AN, Miti ANAS. Evaluation of individual phenolic compounds and antioxidant properties of black , green , herbal and fruit tea infusions consumed in Serbia : spectrophotometrical and electrochemical approaches. *J Food Nutr Res.* 2013;52:12-24

22. Roedig-Penman A, Gordon MH. Antioxidant properties of catechins and green tea extracts in model food emulsions. *J Agric Food Chem.* 1997;45:4267-4270.

23. Gallego MG, Gordon MH, Segovia FJ, Skowyra M, Almajano MP. (2013) Antioxidant Properties of Three Aromatic Herbs (Rosemary, Thyme and Lavender) in Oil-in-Water Emulsions. *J Am Oil Chem Soc.* 2013;90:1559-1568.

24. Pehlivan E, Arslan G, Gode F, Altun T,Özcan M. Determination of some inorganic metals in edible vegetable oils by inductively coupled plasma atomic emission spectroscopy. *Grasas y Aceites.* 2008;59:239-244.

25. Mancuso JR, McClements DJ, Decker E. The effects of surfactant type, pH, and chelators on the oxidation of salmon oil-in-water emulsions. *J Agric Food Chem.*1999;47:4112-4116.

26. Decker EA, Warner K, Richards MP, Shahidi F. Measuring antioxidant effectiveness in food. *J Agric Food Chem*. 2005;53:4303-4310.

27. Tammineni N, Unlü G, Rasco B, Powers J, Sablani S, Nindo C. Trout-skin gelatin-based edible films containing phenolic antioxidants: effect on physical properties and oxidative stability of cod-liver oil model food. *J Food Sci*. 2012;77:342-347.

28. Ahmad M, Benjakul S, Sumpavapol P, Nirmal NP. Quality changes of sea bass slices wrapped with gelatin film incorporated with lemongrass essential oil. *Int J Food Microbiol*. 2012;155:171-178.

29. Jongjareonrak A, Benjakul S, Visessanguan W, Tanaka M. Antioxidative activity and properties of fish skin gelatin films incorporated with BHT and tocopherol. *Food Hydrocoll.* 2008;22:449-458.

3.7. Evaluation of the antioxidant activity of *Betula Pendula Roth.* leaf extract (BP) and its effects on model foods

Abstract

Analysis of the phenolic compounds in *Betula Pendula Roth. (*BP) extract revealed various poly-phenols and their derivatives. The BP ethanol extract showed antioxidant activity in tests with the 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (TEAC) radical cation, the oxygen radical absorbance capacity (ORAC) and the ferric reducing antioxidant power (FRAP) with values of 1.45 mmol Trolox equivalents (TE)/g DW, 2.81 mmol TE /g DW and 1.52 mmol TE/g DW, respectively. The BP methanol extract also exhibited scavenging activity against methoxy radicals generated by the Fenton reaction and measured by Electron Paramagnetic Resonance (EPR). The effect of antioxidant activity measured in beef patties containing 0.1% and 0.3% (w/w) of lyophilised BP stored in a modified atmosphere (80% v/v O_2 and 20% v/v CO_2) was determined. Reductions in lipid oxidation were found in sample treated with BP as manifested by the changes of colour and metmyoglobin concentration. A preliminary study of gelatin based film treated with BP showed that the extract had high antioxidant activity to retard degradation of lipid in muscle food. Thus, the present results indicated that the BP extract can be used as a natural food antioxidant.

Keywords

Betula Pendula Roth., lipid oxidation, MAP, active packaging, antioxidant acivity, food models.

3.7.1 Introduction

Nowadays, there is growing interest of consumers in natural foods and a healthy diet and concern about possible toxicological effects of the synthetic antioxidants used in the food industry. Natural antioxidants gain their activity mainly from polyphenol compounds found in most herbal plants, fruits and vegetables. Previous studies indicate that consumption of plant foods rich in antioxidants is beneficial to health and helps to prevent many diseases such as heart problems, diabetes, neurodegenerative disorder and cancers[1-4]. Moreover, the use of natural antioxidants to replace synthetic antioxidant in model foods such as meat burgers and mayonnaise has been extensively studied in recent years. Incorporating natural antioxidants in muscle foods not only prolonged the shelf life of meat and successfully delayed the oxidation process but it also enhanced the nutritional quality of the meat[5-7].

Recent strategy has focused on the development of active packaging systems based on the incor-poration of natural antioxidants into food packaging formulations[8,9]. This eco-friendly biodegradable packaging not only provides safety benefits to the food, successfully controls food quality and extends the shelf life, but also replaces commercial non degradable plastic which is harmful to the environment. Biopolymers used in film preparation are from protein sources which have many advantages and are abundantly available. Gelatin which is obtained from collagen by hydrolysis is one of the popular ingredients in film making and its advantages include its film forming ability[10].

To date, biodegradable packaging with a natural antioxidant coating has attracted great attention, and numerous research projects are under way in this field[8,11,12]. Natural antioxidants from plants chosen for incorporation into the film contain an abundance of polyphenol constituents. Polyphenols have a wide range of beneficial health effects and their potential for delaying and inhibiting lipid oxidation has been well studied. A gelatin based film coated with 25% (w/w) lemongrass essential oil enhanced the quality and extended the shelf life of sea bass slices[11]. The incorporation of tea polyphenol-loaded chitosan nanoparticles (TPCN) into gelatin in a film improved the antioxidant activity of the gelatin film, whereas phenolic compounds from *Curcuma longa L.* rhizomes extract conferred barrier properties and antioxidant capacity to gelatin based films[8-12].

Betula sp. (Betulaceae) is well known as a birch tree, which has a wide distribution in the north-ern hemisphere from Canada to Japan. Birch, in particular silver birch (Betula Pendula Roth.) has traditionally been important in many countries with all of the plant parts utilized for various medicinal purposes. *Betula Pendula Roth.* (BP) has been used to treat skin diseases especially eczema, infections, inflammations, rheumatism and urinary disorders and the bud oil is also widely used as a tonic and in cosmetic products as an antiseptic particularly in hair products [13-16]. Furthermore, there have been numerous investigations of the benefits of plants for human health over the years[17]. Başer & Demirci (2007) demonstrated the potential of a few Betula species with antifungal, antibacterial and antioxidant effects using various in vitro techniques[18]. It has been reported by Germano et al. (2012) that the BP extract contained many polyphenol constituents such as Catechin, p-Coumaric acid, Myricetin, Quercetin and Kaempferol which are known for their free radical scavenging and antioxidant propertiest[12]. This high content of phenolic compounds may make Betula species suitable for use as antioxidant sources in the food industry.

However, the antioxidant activity of BP leaf extract towards lipid oxidation has not been fully determined yet. Thus, our goals were (1) to identify the phenolic compounds in a BP extract that contribute to the antioxidant activity in the plant using LCMS, (2) to evaluate the antioxidant activity of BP using in vitro assays including FRAP, TEAC, ORAC and EPR scavenging activity and (3) to demonstrate the ability of BP extract to inhibit lipid deterioration in beef meat, either by inclusion in the patty composition or in the formulation of the active packaging.

3.7.2 Materials and Methods

Materials

Commercial dried BP was kindly supplied by Pàmies Hortícoles (Balaguer, Spain), a registered herbal company. All reagents and solvents used were of analytical grade and obtained from Pan-reac (Barcelona, Spain) and Sigma Aldrich (Gillingham, England).

Extraction of Betula Pendula

The preparation, extraction and recovery of BP was carried out according to the method of Azman et al. (2015)[20].

Determination of Phenolic Compound using LCMS

Preparation of BP methanol extracts was carried out as described in the extraction method above. The LCMS analysis procedure was similar to that reported by Skowyra et al. (2014) with small modifications[21]. Compounds were determined using a LC-ESI–QTOF-MS system acquired from Agilent with a 1200 Series HPLC (Wilmington, DE, USA). The LC instrument has two isocratic high pressure mixing pumps, a vacuum degasser unit and a chromatographic oven. The QTOF mass spectrometer was an Agilent 6520 model, furnished with a Dual-Spray ESI source. Compounds were separated in an Agilent Zorbax Eclipse XDB C18 column (100 mm × 2 mm, 3.5 m) connected to a C18 (4 mm × 2 mm) guard cartridge from Phenomenex (Torrance, CA, USA). Ultrapure water (A) and acetonitrile (B), both containing 0.1% formic acid, were used as mobile phases applying the following gradients: 0–10 min, 3% B; 15-17 min, 100% B; 11 min 3% B. The mobile phase flow was 0.2 mL min−1, the injection volume for standards and sample extracts was 10 µL and the column temperature was set at 30◦C. The mobile phase flow was 0.2 mL/min, using the gradients: 0–10 min, 3% B; 10–25 min, 100% B; 27–38 min, 3% B. Nitrogen (99.999%), was used as nebulizing (35 psi) and drying gas (330 °C, 10 °C/min) (Carburos Me-tálicos, A Coruña, Spain). The QTOF instrument was operated in the 2 GHz mode (Extended Dynamic Range, mass resolution from 4500, at m/z 100, to 11,000, at m/z 900) and compounds were ionized in positive ESI, applying capillary and fragmentor voltages of 3500 and 160 V, respectively. The Mass Hunter Workstation software was used to control all the acquisition pa-rameters of the LC-ESI-QTOF-MS system and also to process the obtained data. Full scan MS spectra were acquired in the range from 100 to 1700 m/z units. The identification of polyphenol composition was based on the accurate masses, isotopic abundances and spacing of signals in their cluster of ions ([M + H]+), obtained in the MS mode, as well as, on their MS/MS fragmen-tation patterns and the exact mass of product ions.

Determination of the Total Phenolic Content (TPC)

The Folin-Ciocalteu method was used to determine the total phenolic content (TPC) as reported by Santas et al. (2008)[22].

Determination of free radical scavenging activity assays

a. *In vitro* antioxidant capacity determination

Three different methods were used for the evaluation of the antioxidant activity of the extracts: 2,2′-azino-bis-(3-ethylbenzthiazoline)-6-sulphonic acid ($ABTS^{\bullet+}$) TEAC assay[23], Oxygen Radical Absorbance Capacity (ORAC) assay[21] and Ferric Reducing Antioxidant Power (FRAP) method[24]. Results were expressed as μM of Trolox equivalent (TE) per gram of dry weight of plant (DW).

b. Electron Paramagnetic Resonance (EPR) Spectroscopy Radical Scavenging assay

EPR radical scavenging activity was measured following the method of Azman et al. (2014)[25]. The extraction was executed in MeOH with 1:10 (w/v) ratio and the soluble concentration of BP was determined by lyophilization. A spin-trapping reaction mixture consisted of 100 μL of DMPO (35 mM); 50 μL of H_2O_2 (10 mM); 50 μL BP extract at different concentrations or 50 μL of ferulic acid used as reference (0−20 g/L) or 50 μL of pure MeOH used as a control; and, finally, 50 μL of $FeSO_4$ (2 mM), added in this order. The final solutions (125 μL) were passed to a narrow (inside diameter = 2 mm) quartz tube and introduced into the cavity of the EPR spectrometer. The spectrum was recorded 10 min after the addition of the FeSO4 solution, when the radical adduct signal is greatest. X-band EPR spectra were recorded with a Bruker EMX-Plus 10/12 spectrometer under the following conditions: microwave frequency, 9.8762 GHz; microwave power, 30.27 mW; centre field, 3522.7 G; sweep width, 100 G; receiver gain, 5.02 × 104; modulation frequency, 100 kHz; modulation amplitude, 1.86 G; time constant, 40.96 ms; conversion time, 203.0 ms.

Determination of antioxidant activity in food model

a. Preparation of beef patties

The initial preparation method of beef patties was adapted from Azman et al. (2014)[26]. The meat was divided into 4 batches and was mixed with 1.5 % NaCl and either i) control (no addition), ii) 0.1 % BHT, iii) 0.1 % lyophilized BP, iv) 0.3 % lyophilized BP and moulded into smaller portions (about 20 g each). The modified atmosphere was maintained by using polystyrene B5-37 (Aerpack) trays which were placed in BB4L bags (Cryovac) of low gas permeability (8–12 cm^3 m^-2 per 24 h). The air in the trays was flushed with 80:20 (v/v) O_2:CO_2 by EAP20 mixture (Carburos Metalicos, Barcelona) and the trays were packaged. Samples were stored in the dark at 4 ± 2 ^{o}C for 10 days to analyse the extent of oxidation by the thiobarbituric acid reactive substances (TBARS) method, % metmyoglobin, colour, pH and microbial quality. Every measurement was carried out in triplicate each day for 10 days (except for microbiological analysis which was done every 3 days).

b. Thiobarbituric acid reacting substances (TBARS)

Lipid peroxidation was taken as an indicator of oxidative damage and was assessed by measuring the content of thiobarbituric acid reactive substances (TBARS)[27]. The modified method was adapted from Azman et al. (2014)[26]. All results were reported in mg malonaldehyde per kg of sample (mg MDA/kg sample).

c. Colour measurement and metmyglobin percentage

Objective measurements of colour were performed using a CR 400 colorimeter (Minolta, Osaka, Japan). Each patty was cut and the colour of the slices was measured three times for each point. A portable colorimeter with the settings: pulsed xenon arc lamp, 0° viewing angle geometry and aperture size 8 mm was used to measure meat colour in the CIELAB space (Lightness, L*; red-ness, a*; yellowness, b*). Before each series of measurements, the instrument was calibrated using a white ceramic tile. The metmyoglobin percentage was determined by the method devel-oped by Xu et al. (2010). All sample measurements were carried out in triplicate.

d. Development of gelatin-film with antioxidant coating

The fabrication and characterization of the gelatin based film with antioxidant coating was based on the method of Bodini et al. (2013)[29]. Whilst the filmogenic solution was cooled after the solubilization of sorbitol, 0.1% (w/w) of BP extract / gelatin and 0.1% (w/w) BHT/gelatin was added. The lipid degradation was measured using the TBARS method as mentioned above.

Statistical Analysis

A one-way analysis of variance (ANOVA) was performed using Minitab 16 software program (α=0.05). The results were presented as mean values ($n \geq 3$).

3.7.3 Results and Discussion

Extraction yield, total phenolic content (TPC) and antioxidant activity

A number of studies have found that the antioxidant activity of BP extracts correlates with the phenolic content, and thus the identification of the phenolic compounds in the plant extract may reveal compounds responsible for its antioxidant activity in various assays[19,30,31].

Table 1 : Polyphenol composition identified in methanol extract of BP using LCMS.

No.	t_R (min)	Molecular Formula	$[M\text{-}H]^-$	Proposed Compound
1	14.2	$C_{15}H_{14}O_6$	298.0718	Catechin
2	17.3	$C_9H_8O_3$	163.0401	p-Coumaric acid
3	29.31	$C_{15}H_{10}O_8$	317.0303	Myricetin
4	31.6	$C_{15}H_{10}O_7$	301.0354	Quercetin
5	32.8	$C_{15}H_{12}O_5$	271.0612	Naringenin

There were 5 polyphenol constituents found in the methanol extract of BP (Table 2) which had all been reported previously[19,32]. Moreover, previous authors found more than 26 polyphenol constituents in the methanol extract including Kaempferol and its derivatives. Studies of the betula spp constituents including BP have been reported by few authors[17,19,]. The chemical composition of flavonoids as the main polyphenolic group of constituents in birch leaves has been investigated quite extensively for several years. For example, Raal et al. (2015)[33] developed the use of phenolic compounds as chemical indicators of a few birch species while Evans (2000)[34] identified the chemical structures of quercetin and hyperoside as the main flavonoids in a BP extract. Among the components listed above, quercetin has been reported as the main active ingredient of birch leaves and a possible synergistic action of several flavonoids and phenolic components has also been described in BP active ingredients[17]. Isolation of flavonoid constituents from BP was investigated due to their many pharmacological benefits in human health.

Analysis of Total Polyphenols and Free Radical Activity Assays

a. Total phenolic content and *in-vitro* antioxidant activity

On average, BP extracted with 50% ethanol produced a greater dry weight of soluble extract compared to 75% and 90% ethanol. Ethanol was selected for use in the extraction solvent since the alcohol is recognized as a GRAS (Generally Recognised as Safe) material which can be used for applications in the food industry[35]. Ethanol is also effective in the extraction of flavonoids and their glycosides, catechols and tannins from raw plant materials. Raal et al. (2015) reported that 20% of ethanol gives the highest polyphenol yield analyzed by net area under the curve (AUC) in HPLC chromatograms[33].

Table 2: Soluble concentration, TPC and Antioxidant activity of BP extract.

Activity	Extraction solvent		
Betula Pendula	90:10 EtOH:H_20	75:25 EtOH:H_20	50:50 EtOH:H_20
Soluble concentration (g/l)	19.83 ± 0.05^{a}	20.1 ± 0.02^{b}	22.6 ± 0.05^{c}
Total phenolic content (mg GAE / g DW)	10.8 ± 0.05^{a}	9.11 ± 0.03^{b}	11.23 ± 0.02^{ac}
FRAP (mmol of TE / g DW)	1.59 ± 0.02^{a}	1.06 ± 0.06^{b}	1.52 ± 0.01^{ac}
TEAC (mmol of TE / g DW)	1.27 ± 0.05^{a}	1.36 ± 0.03^{ab}	1.45 ± 0.02^{bc}
ORAC (mmol of TE / g DW)	1.56 ± 0.05^{a}	1.67 ± 0.05^{b}	2.81 ± 0.03^{c}

*Mean value n = 3 and the standard deviation for each assay is less than 5%. Gallic Acid Equivalent (GAE), Trolox Equivalent (TE), Dry Weight (DW). $^{a-c}$: Means within a row with different letters are significantly different ($P<0.05$).

Table 2 shows that 50% ethanol extract gave significantly higher value of phenol content com-pared to 90% and 75% ethanol extract ($p<0.05$). Generally, BP extracted with 50% ethanol showed higher antioxidant activity values in the ORAC assays ($p<0.05$). Mashentseva et al. (2011) demonstrated higher values of phenolic content and TEAC values of the ethanol extract than with petroleum ether by the Soxhlet extraction[36]. The ORAC assay gave the highest antioxidant activity values compared to the FRAP and TEAC assays, and showed the scavenging activity of the extract towards peroxy radicals (OOH•) generated in the assay. The literature has reported the antioxidant activity of BP analyzed using the DPPH method, nitric oxide scavenging activity and the reducing power assay[36]. To the best of our knowledge, this is the first report of the antioxidant activity of extracts from BP assessed using the ORAC methods.

b. EPR scavenging radical assay

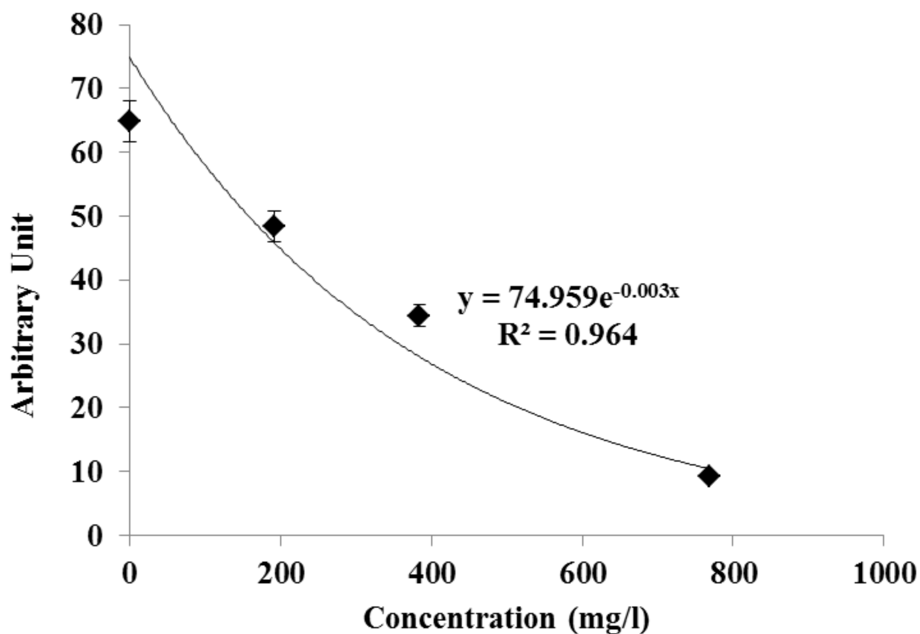


Figure 1. Area of the Electron paramagnetic resonance (EPR) spectra of the radical adduct DMPO-OCH3 generated from a solution of H_2O_2 [2 mM] and $FeSO_4$ [0.04 mM] with DMPO [14 mM] as spin trap in MeOH as solvent. The area of the EPR signal is plotted against concentration of BP methanolic extracts.

The EPR radical scavenging method has been developed by Azman et al. (2014) to evaluate the free methoxy radical ($CH3O^•$) generates by Fenton reaction and this was applied to the BP extract[25]. Figure 1 showed the decreasing signal of EPR with increasing concentration of BP extract. The free radical scavenging activity of BP extracts against methoxy ($CH3O^•$) radical was investigated by a competitive method in the presence of DMPO as spin trap, using EPR spectroscopy. The $CH3O^•$ radical generated according to the Fenton procedure has a relatively short half-life but was identified by EPR because of its ability to form a stable nitroxide adduct with DMPO, DMPO-OCH_3 (hyperfine splitting constants, aN = 13.9 G and aH = 8.3 G). This stable DMPO-OCH_3 compound can be detected by the double integration value of the signal from the EPR spectrum. The BP extract at different concentrations competed with the spin trap DMPO in the scavenging of methoxy radicals. Thus, the antioxidant decreased the amount of radical adducts and, accordingly, decreased the intensity of the EPR signal. The best fitting with

intensity of EPR signal was shown as exponential function (Figure 1) that, if concentration values are in g/L, corresponds to equation (1):

$$y = 74.959e\text{-}0.003x;\ R_2 = 0.964 \qquad (1)$$

The graph indicates the exponential relationship of the decrease in signal in the spectrum as the concentration of BP increased. This study confirmed that the scavenging activity of the BP ex-tracts containing polyphenol constituents could be measured by the decrease of the intensity of the spectral bands of the adduct DMPO-OCH_3 in the EPR spectrum. The radical scavenging activity of BP has been determined by various methods previously including the DPPH radical scavenging activity and the Ferrous ion chelating activity has also been reported[32]. However, this study is the first report of the measurement of BP extract potential as act as an antiradical compound by scavenging methoxy radicals generated from the Fenton reaction.

Antioxidant activity in Food Model

a. Color and % Metmyoglobin

Meat colour is one of the most important parameters that indicate meat quality. The colour values representing lightness (L*), redness (a*), and yellowness (b*) are shown in Table 3. The initial mean lightness (CIE L*) was 29.773 ± 0.866, and the control sample showed the highest value of L* at the end of 10 days storage. The L* values showed the increase in lightness of the meat due to the increased fat percentage but the redness was reduced. At the end of the storage period, samples containing 0.3% BP demonstrated the lowest value of L* while the sample containing BHT and the sample containing 0.1% BP displayed similar values. Our results were in good agreement with those of Triki et al. (2013) and Heyes et al. (2010) who showed that reduced fat in muscle food tends to reduce L* and increase a* when compared to control fat products, while redness decreased and lightness increased with storage time[6,37]. Few authors have reported that lipid oxidation results in a decrease in redness[38,39]. Beef patties containing 0.3% BP maintained a more red colour (a*) throughout the display, whereas the control beef patties became discoloured from 6 days onwards in the MAP. All samples showed a significant decrease of red colour after 8 days storage ($p<0.05$).

Table 3: Effect of BP extract and BHT on instrumental colour value (L*, a*, b*) of beef patties during 10 days of refrigerated storage at 4°C. (Mean±SE).

Assay	Sample	Days of storages					
		0	2	4	6	8	10
L*	Control	29.773 ± 0.866^{a1}	38.676 ± 1.499^{a2}	29.197 ± 0.326^{a1}	37.097 ± 1.232^{a2}	37.228 ± 0.447^{a2}	45.612 ± 2.217^{a3}
	0.1% BHT	29.773 ± 0.866^{a1}	32.857 ± 1.780^{b2}	30.550 ± 0.967^{b3}	35.436 ± 1.062^{b4}	37.992 ± 1.192^{a5}	40.582 ± 0.462^{b6}
	0.1% BP	29.773 ± 0.866^{a1}	34.475 ± 1.732^{c2}	30.915 ± 1.518^{b3}	37.297 ± 0.941^{a4}	41.023 ± 1.081^{b5}	40.980 ± 0.462^{b5}
	0.3 % BP	29.773 ± 0.866^{a1}	28.420 ± 1.580^{d1}	36.448 ± 1.744^{c2}	33.117 ± 1.380^{c3}	36.475 ± 1.999^{c2}	36.230 ± 1.904^{c2}
a*	Control	7.490 ± 0.080^{a1}	6.765 ± 0.286^{a2}	6.540 ± 0.328^{a2}	6.265 ± 0.425^{a2}	4.724 ± 0.376^{a3}	0.887 ± 0.010^{a4}
	0.1% BHT	7.490 ± 0.080^{a1}	8.183 ± 0.426^{b2}	9.282 ± 0.282^{b3}	7.050 ± 0.260^{b1}	7.364 ± 0.406^{b1}	2.865 ± 0.010^{b4}
	0.1% BP	7.490 ± 0.080^{a1}	7.368 ± 0.234^{c1}	8.742 ± 0.130^{c2}	7.252 ± 0.321^{b1}	5.643 ± 0.150^{c3}	1.245 ± 0.040^{b4}
	0.3 % BP	7.490 ± 0.080^{a1}	9.165 ± 0.185^{d2}	8.685 ± 0.340^{c3}	8.380 ± 0.176^{c3}	6.748 ± 0.130^{d4}	2.900 ± 0.010^{b5}
b*	Control	7.417 ± 0.320^{a1}	4.864 ± 0.065^{a2}	7.666 ± 0.362^{a1}	8.550 ± 0.090^{a3}	9.947 ± 0.140^{a4}	6.767 ± 0.160^{a5}
	0.1% BHT	7.417 ± 0.320^{a1}	6.680 ± 0.001^{b2}	8.403 ± 0.150^{b3}	8.394 ± 0.070^{a3}	8.379 ± 0.060^{b3}	2.101 ± 0.440^{b4}
	0.1% BP	7.417 ± 0.320^{a1}	6.360 ± 0.230^{b2}	8.962 ± 0.526^{b3}	8.408 ± 0.120^{a3}	8.764 ± 0.020^{b3}	2.118 ± 0.330^{b4}
	0.3 % BP	7.417 ± 0.320^{a1}	9.186 ± 0.508^{c2}	10.186 ± 0.270^{c3}	7.903 ± 0.180^{b1}	5.000 ± 0.060^{a4}	6.901 ± 0.200^{a5}

Control: 1.5% salt (w/w); 0.1% BHT: 1.5% salt with 0.1% BHT (w/w); 0.1% BP: 1.5% salt with 0.1% BP (w/w) 0.3% BP: 1.5% salt with 0.3% BP (w/w). $^{a-d}$: Means within a row with different letters are significantly different (P<0.05). $^{1-6}$: For each attribute, means within a column with different letters are significantly different (P<0.05). Mean value n = 6 and the standard deviation for each assay is less than 5%.

Consumers scrutinize meat freshness by its visual redness and the red colour reflects the state of oxidation of the pigment in the meat[40]. At the end of storage, the a* value of the control sample meat product was significantly lower ($p \leq 0.05$) than that of the other samples tested. Therefore the natural plant extract affected meat colour and was potentially useful in delaying the oxidation and discoloration of the meat product. In general, no significant differences ($p > 0.05$) were observed in the b* values of samples during storage. The present findings seem to be consistent with other research which found that yellowness in meat patties is not influenced by time of storage and packaging conditions[37,41]. Meanwhile, Muthukumar et al (2014) and Rojas and Brewer (2007) reported that the b* value of cooked and raw patties with antioxidants showed a more gradual reduction compared to control during storage[42,43].

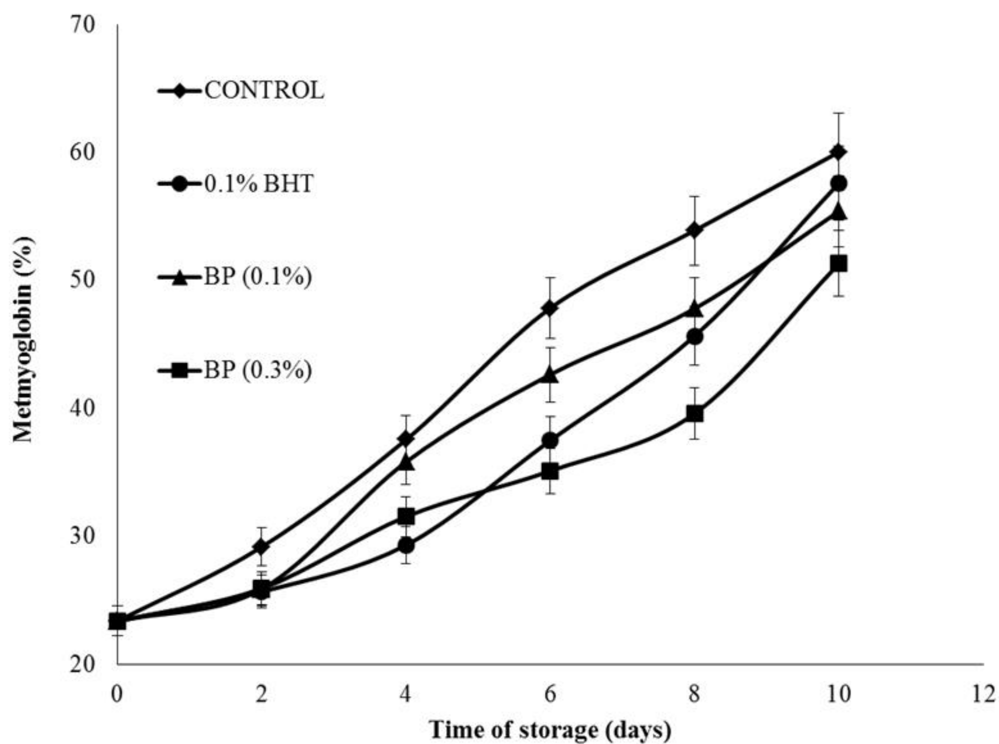


Figure 2. Effects of BP extract and BHT on metmyoglobin changes of beef patties during 10 days of refrigerated storage at 4°C. (Mean±SE). Control: 1.5% salt (w/w); 0.1% BHT: 1.5% salt with 0.1% BHT (w/w); 0.1% BP: 1.5% salt with 0.1% BP (w/w) 0.3% BP: 1.5% salt with 0.3% BP (w/w). Mean value n = 3 and the standard deviation for each assay is less than 5%.

The changes in BP treated samples and BHT on MMb formation during storage are presented in Figure 2. Both control and treated samples, upon storage showed an increase (P<0.001) in MMb formation (p>0.05). The MMb value relates to the instrumental colour a* values. Free radicals produced by lipid oxidation in meat are susceptible to initiate the reaction of oxidizing oxymyo-globin (red colour) to metmyoglobin (brown colour) which results in the discolouration of meat during storage. Samples treated with antioxidant can reduce the oxidation of metmyoglobin by scavenging hydroxyl radicals produced from oxidation of oxymyoglobin. This finding is sup-ported by several authors who observed that the decrease of colour in muscle food is influenced by the decrease in metmyoglobin concentration during storage[28-44].

b. TBARS analysis in beef patties

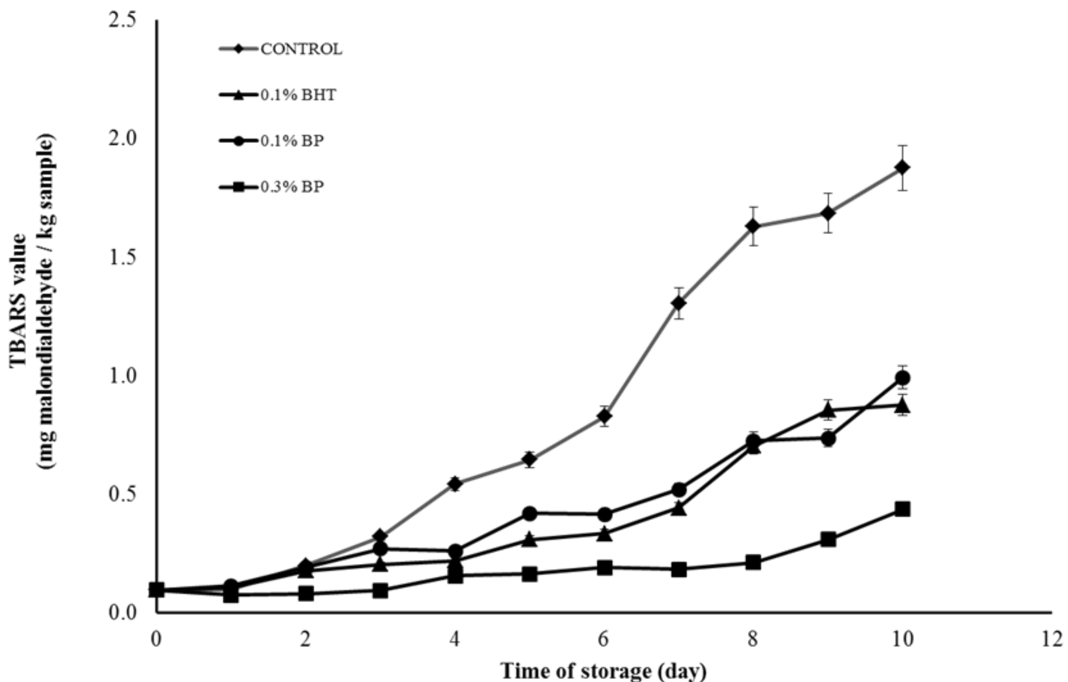


Figure 3: Changes in TBARS values (mg malodialdehyde/kg sample) of control and sample containing different concentrations (0.1% and 0.3% w/w) of BP extract in MAP atmosphere during 10 days storages at 4 ± 1^{O}C without light. Each sample was measured in triplicate and the average standard deviation for each sample was less than 5%.

The effect of different concentrations of BP and BHT on TBARS values in meat patties during the 10 days storage at 4 °C is shown in Figure 3. The TBARS values of all samples treated with antioxidant were significantly ($p<0.05$) reduced compared with the control throughout the stor-age period in the MAP atmosphere. However, the BHT sample and 0.1% BP sample were not significantly different throughout storage. This showed that 0.1% BP would be sufficient to in-hibit the generation of MDA significantly and was similar in activity to 0.1% BHT in MAP. 0.3% (w/w) BP displayed the lowest TBARS values with less than 1.0 mg malondialdehyde/kg sample at the end of the storage time; and this indicates that the sample experienced a strong antioxidant effect. The use of 0.1% BHT was added as comparison and is an effective dose within the legal limitation for use in food[45]. The effect of BP extract towards lipid oxidation in meat has never been reported. Reduction of lipid oxidation with BP in meat patties in MAP could be attributed to the presence of polyphenols, rich in catechins as well as other flavonoids. The active properties of BP have been reported by many authors previously[19,30,33]. Our LCMS study also showed the presence of phenolic acids in the extract which may contribute to inhibition of lipid oxidation in the meat. The antioxidant activity of phenolic compounds is closely related to the hydroxyl group linked to the aromatic ring which is capable of donating hydrogen atoms and neutralizing free radicals. This mechanism blocks further degradation of active oxidised compounds such as MDA, which can be measured by the TBARS method[38]. The study confirmed the potential of BP extract to inhibit lipid degradation in beef patties.

c. TBARS analysis in active packaging

Meat packed under films coated with antioxidants, namely BP and BHT applied at 0.1 %, expe-rienced a decrease of lipid oxidation compared to the control sample. At the first days of assay, no significant differences between all samples tested were observed. The significant differences in TBARS values started at day 2 ($p<0.05$) and differences continued until 17 days storage. The samples containing synthetic and natural antioxidant showed a tendency to be marginally differ-ent ($p<0.1$) throughout the storage period. The TBARS values of meat packed under films with 0.1% of antioxidant product also showed a significant reduction in lipid oxidation of the meat throughout the entire storage period compared to control. The increased food stability of samples containing BP and BHT can

be seen in Figure 4, and it can be pointed out that the marginal dif-ference between samples containing BP extract and BHT, which is recognised as an important antioxidant, demonstrates the effectiveness of the natural antioxidant as an alternative preserva-tive for the food industry. The literature reports that BP contained many phenolic compounds that contribute to its strong antioxidant activity as shown by several assays[17,19,30].

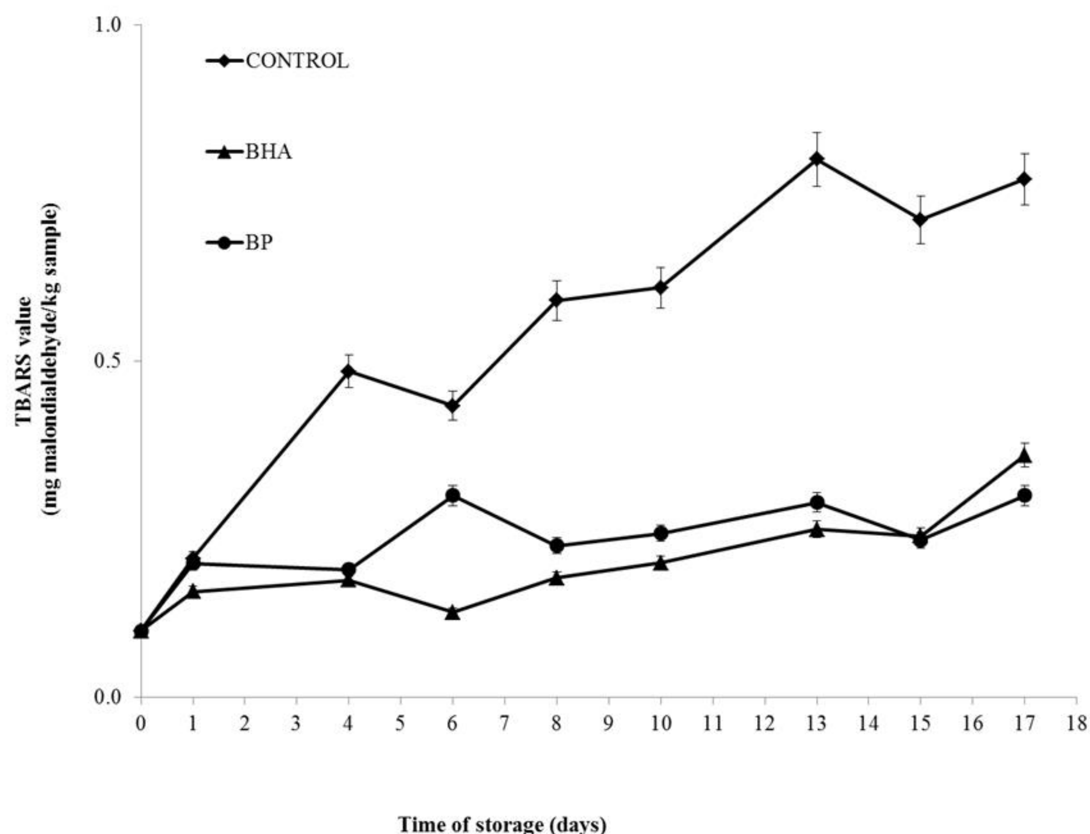


Figure 4: Changes in TBARS values (mg malodialdehyde/kg sample) of control and sample containing and 0.1% w/w BHT and BP extract in MAP atmosphere during 17 days storages at 4 ± 1^{O}C without light. Each sample was measured in triplicate and the average standard deviation for each sample was less than 5%.

Radone et al. (2014) demonstrated that the phenolic components of the BP extracts (which include hyperoside and chlorogenic acid) possess strong antioxidant activity when measured by the HPLC-FRAP post column assay. Our LCMS analysis of the BP extract

showed that a few phenolic constituents such as catechin and p-coumaric acid exhibited antioxidant activity that has the potential to contribute to many pharmacological benefits to humans including antioxidant, and anticancer effects[46-47]. Moreover, many constituents present in the BP extract correlated significantly with antioxidant activity measured by ORAC and TEAC assays and may play important roles in the detoxification of endogenous compounds in humans[48].

Thus, this study set out with the aim of assessing the potential effects of BP extract in delaying lipid oxidation in a food model. Beside the promising result of antioxidant activity and the pres-ence of polyphenols in the extract, this is a preliminary study of the properties of gelatin based film containing BP extract for environmental friendly packaging for foods. Duthie et al. (2013) demonstrated the presence of phenolic acids including p-coumaric acid measured using LCMS in chicken patties mixed with vegetable powders[49]. The compound found in Duthie et al. (2013) work is relevant to our findings for the BP extract that gave a good protective effect to the meat patties. Furthermore, our work also showed the efficacy of gelatin-based films treated with 0.1% BP extract.

3.7.4 Conclusion

The BP extract prepared with 50% aqueous ethanol showed a high phenolic content and antioxidant activity measured by the FRAP, TEAC and ORAC assays. The various polyphenol constituents present in the BP extract contribute to the antioxidant activity and total polyphenol content. The BP extract showed scavenging ability towards methoxy radicals generated by the Fenton Reaction assessed by EPR. Lyophilized BP (0.1% and 0.3 % w/w) can be applied as an antioxidants in meat patties since these extracts inhibited lipid oxidation significantly in samples packed under MAP. 0.3% BP decreased the discoloration of meat and the brown colour measured by the metmyoglobin assay significantly. A preliminary study of the effect of gelatin based film coated with 0.1% (w/w) BP showed that it significantly delayed degradation of lipid in meat ($p<0.05$). Therefore, this study confirmed that BP as a source of antioxidants has potential to be used by the food industry.

References

1. Aliakbarlu J and Tajik H. Antioxidant and Antibacterial Activities of Various Extracts of Borago Officinalis Flowers. *J Food Process Preserv.* 2012;36*:*539–544.

2. Decker E, Warner K, Richards MP, Shahidi F. Measuring antioxidant effectiveness in food. *J Agr Food Chem*. 2005;53:4303–10.

3. Halliwell, B. Antioxidants in human health and disease. *Annu. Rev. Nutr.*1996;16:33–50.

4. Pandey KB and Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid Med Cell Longev.* 2009;2:270–8.

5. Doménech-Asensi G, García-Alonso FJ, Martínez E, Santaella M, Martín-Pozuelo G, Bravo S, Periago MJ. Effect of the addition of tomato paste on the nutritional and sensory properties of mortadella. *Meat Sci.* 2013;93:213–9.

6. Hayes JE, Stepanyan V, Allen P, O'Grady MN, Kerry JP. Effect of lutein, sesamol, ellagic acid and olive leaf extract on the quality and shelf-life stability of packaged raw minced beef patties. *Meat Sci.* 2010;84: 613–20.

7. Sanchez-Escalante A, Djenane D, Torrescano G, Beltran JA, Roncales P. Antioxidant Action of Borage, Rosemary, Oregano, and Ascorbic Acid in Beef Patties Packaged in Modified Atmosphere. *J Food Sci*. 2003;68:339–344.

8. Bao S, Xu S, Wang Z. Antioxidant activity and properties of gelatin films incorporated with tea polyphenol-loaded chitosan nanoparticles. *J. Agric. Food. Chem.* 2009;89:2692–2700.

9. Gómez-Estaca J, Montero P, Giménez B, Gómez-Guillén MC. Effect of functional edible films and high pressure processing on microbial and oxidative spoilage in cold-smoked sardine (Sardina pilchardus). *Food Chem.* 2007;105:511–520.

10. Ali Y, Benjakul S, Prodpran T, Sumpavapol P. Food Hydrocolloids Properties and antimicrobial activity of fish protein isolate / fish skin gelatin fi lm containing basil leaf essential oil and zinc oxide nanoparticles. *Food Hydrocolloids.* 2014;41:265–273.

11. Ahmad M, Benjakul S, Sumpavapol P, Nirmal NP. Quality changes of sea bass slices wrapped with gelatin film incorporated with lemongrass essential oil. *Int J Food Microbiol.*2012;155:171–178.

12. Bitencourt CM, Fávaro-Trindade CS, Sobral PJ, Carvalho R. Gelatin-based films additivated with curcuma ethanol extract: Antioxidant activity and physical properties of films. *Food Hydrocolloids*. 2014;40:145–152.

13. Jäger S, Laszczyk MN, Scheffler A. A preliminary pharmacokinetic study of betulin, the main pentacyclic triterpene from extract of outer bark of birch (*Betulae alba cortex*). *Molecules*, 2008;13:3224–3235.

14. Jine Y, Lis M, Szczypka M, Obmińska-Mrukowicz B. Influence of betulinic acid on lymphocyte subsets and humoral immune response in mice. *Pol J Vet Sci.* 2012;15:305–313.

15. Meyer-Hoffert U, Brasch J. Allergic contact dermatitis caused by betulin-containing triterpene extract from the outer bark of birch (*Betula alba*). *Contact Dermatitis*. 2013;68:382–383.

16. Rosado T, Bernardo P, Koci K, Coelho AV, Robalo MP, Martins LO. (). Methyl syringate: An efficient phenolic mediator for bacterial and fungal laccases. *Bioresour. Technol.* 2012;124:371–378.

17. EMEA. (2014). European Medicines Agency, Retrieved from http://www.ema.europa.eu/ema/

18. Başer KHC, Demirci B. Studies on Betula essential oils. *Arkivoc*. 2007;vii:335–348.

19. Germanò MP, Cacciola F, Donato P, Dugo P, Certo G, D'Angelo V, Mondello L, Rapisarda A. Betula pendula leaves: Polyphenolic characterization and potential innovative use in skin whitening products. *Fitoterapia*. 2012;83:877–882.

20. Azman NAM., Gallego M, Juliá L, Fajari L, Almajano MP. The Effect of Convolvulus arvensis Dried Extract as a Potential Antioxidant in Food Models. *Antioxidants*. 2015;4:170–184.

21. Skowyra M, Falguera V, Gallego G, Peiró S, Almajano MP. Antioxidant properties of aqueous and ethanolic extracts of tara (Caesalpinia spinosa) pods in vitro and in model food emulsions. *J. Sci. Food Agr.* 2013;94:911-8.

22. Santas J, Carbo R, Gordon M, Almajano M. Comparison of the antioxidant activity of two Spanish onion varieties. *Food Chem.* 2008;107:1210–1216.

23. Almajano MP, Carbó R, Jiménez JAL, Gordon MH. Antioxidant and antimicrobial activities of tea infusions. *Food Chem.* 2008;108:55–63.

24. Gallego MG, Gordon MH, Segovia FJ, Skowyra M, Almajano MP. Antioxidant Properties of Three Aromatic Herbs (Rosemary, Thyme and Lavender) in Oil-in-Water Emulsions. *J. Am. Oil Chem. Soc.* 2013;90:1559–1568.

25. Azman NAM, Peiró S, Fajarí L, Julià, L, Almajano MP. Radical scavenging of white tea and its flavonoid constituents by electron paramagnetic resonance (EPR) spectroscopy. *J. Agric. Food. Chem.* 2014;62:5743–8.

26. Azman NAM, Gordon MH, Skowyra M, Segovia F, Almajano MP. Use of lyophilised and powdered Gentiana lutea root in fresh beef patties stored under different atmospheres. *J. Sci. Food Agr.* 2014. Epub ahead of print.

27. Grau A, Guardiola F, Boatella J, Barroeta A, Codony R. Measurement of 2-thiobarbituric acid values in dark chicken meat through derivative spectrophotometry: influence of various parameters. *J. Agric. Food. Chem.* 2000;48:1155–9.

28. Xu Z, Tang M, Li Y, Liu F, Li X, Dai R. Antioxidant properties of Du-zhong (Eucommia ulmoides Oliv.) extracts and their effects on color stability and lipid oxidation of raw pork patties. *J. Agric. Food. Chem.* 2010;58:7289–96.

29. Bodini RB, Sobral PJ, Favaro-Trindade CS, Carvalho R. Properties of gelatin-based films with added ethanol–propolis extract. *Lwt-Food Sci Technol*. 2013;51:104–110.

30. Bertrams J, Kunz N, Müller M. Phenolic compounds as marker compounds for botanical origin determination of German propolis samples based on TLC and TLC-MS. *J Appl Bot Food Qual*. 2013;153:143–153.

31. Raudonė L, Raudonis R, Janulis V, Viškelis P. Quality evaluation of different preparations of dry extracts of birch (*Betula pendula Roth*) leaves. *Nat Prod Res.* 2014;28, 1645–1648.

32. Germanò MP, Cacciola F, Donato P, Dugo P, Certo G, D'Angelo V, Mondello L, Rapisarda A. Betula pendula Roth leaves: gastroprotective effects of an HPLC-fingerprinted methanolic extract. *Nat Prod Res*. 2013;27:1569–1575.

33. Raal A, Boikova T, Püssa T. Content and Dynamics of Polyphenols in Betula spp . Leaves Naturally Growing in Estonia. *Rec. Nat. Prod.* 2015;1:41–48.

34. Evans WC. 2000. Trease and Evans pharmacognosy’, 15th ed. Saunders, Edinburgh

35. Fernández-Agullóa A, Pereira E, Freire MS, Valentão P, Andrade PB, González-Álvarez J, Pereira JA. Influence of solvent on the antioxidant and antimicrobial properties of walnut (Juglans regia L.) green husk extracts. *Ind Crop Prod*. 2013;42:126–132.

36. Mashentseva AA, Dehaen W, Seitembetov TS, Seitembetova AJ. Comparison of the antioxidant activity of the different betula pendula roth. Extracts from northern Kazakhstan. *J. phytol.* 2011;3:18-25.

37. Triki M, Herrero M, Rodríguez-Salas L, Jiménez-Colmenero F, Ruiz-Capillas C. Chilled storage characteristics of low-fat, n-3 PUFA-enriched dry fermented sausage reformulated with a healthy oil combination stabilized in a konjac matrix. *Food Control*. 2013;31:158–165.

38. Kim SJ, Cho AR, Han J. Antioxidant and antimicrobial activities of leafy green vegetable extracts and their applications to meat product preservation. *Food Control*. 2013;29:112–120.

39. Pérez-Álvarez JA and Fernández-López J. *Color Characteristics of Meat and Poultry Analysis, Handbook of Processed Meats and Poultry Analysis*. (In L. Nollet & FT ed.). Boca Raton: CRC Press, 2009. 355-373.

40. Kim SJ, Min SC, Shin HJ, Lee YJ, Cho AR, Kim SY, Han J. Evaluation of the antioxidant activities and nutritional properties of ten edible plant extracts and their application to fresh ground beef. *Meat Sci.* 2013;93:715–22.

41. Cachaldora A, García G, Lorenzo JM, García-Fontán MC. Effect of modified atmosphere and vacuum packaging on some quality characteristics and the shelf-life of "morcilla", a typical cooked blood sausage. *Meat Sci*. 2013;93:220–5.

42. Muthukumar M, Naveena BM, Vaithiyanathan S, Sen R, Sureshkumar K. Effect of incorporation of Moringa oleifera leaves extract on quality of ground pork patties. *J Food Sci Tech*. 2012;51:1–9.

43. Rojas MC and Brewer MS. Effect of natural antioxidants on oxidative stability of frozen, vacuum-packaged beef and pork. 2007;31:173–188.

44. Formanek Z, Lynch A, Galvin K, Farkas J, Kerry JP. Combined effects of irradiation and the use of natural antioxidants on the shelf life stability of over wrapped minced beef. *Meat Sci.* 2003.63;433-440.

45. Post R, Buda, C, Canavan J, Duncan-Harrington T, Jones B, MurphyJenkins R, Myrick T, Wheeler M, White P, Yoder L, Kegley M. A Guide To Federal Food Labeling Requirements for Meat, Poultry and Egg Products Food Safety and Inspection Serv. *Meat, Poultry*, USDA (3), 2007. 241–256.

46. Kiliç, I and Yeşiloğlu Y. Spectroscopic studies on the antioxidant activity of p-coumaric acid. *Spectrochim. Acta Mol. Biomol.* 2013;115:719–24.

47. Nithiyanantham S, Siddhuraju P, Francis G. A promising approach to enhance the total phenolic content and antioxidant activity of raw and processed Jatropha curcas L. kernel meal extracts. *Ind Crop Prod*. 2013;43:261–269.

48. Yeh CT and Yen GC. Effects of phenolic acids on human phenolsulfotransferases in relation to their antioxidant activity. *J. Agric. Food. Chem.* 2013;51:1474–9.

49. Duthie G, Campbell F, Bestwick C, Stephen S, Russell W. Antioxidant effectiveness of vegetable powders on the lipid and protein oxidative stability of cooked Turkey meat patties: implications for health. *Nutrients.* 2013;5:1241–52.

CHAPTER 4

CHAPTER 4: CONCLUSIONS

4.1 Overall Conclusion

In summary, reactive oxygen and oxidative stress play a major role in the development of human diseases such as diabetes, degenerative diseases, Parkinson's disease, heart diseases, cancer and many more. Many studies have identified natural sources of antioxidants in plants, fruits and herbs, all of which are potent preventives of various diseases. Thus, this has triggered enormous interest from researchers worldwide in the bioavailability of endogenous and exogenous natural antioxidants as functional foods to diminish disease. Protection against free radicals can be supplied by sufficient intake of dietary antioxidants. With these recent developments, the formulation of natural antioxidants into food products that are effective and acceptable on the market is very challenging.

Therefore, in this study, we analysed 5 plants (white tea leaves, yellow Gentian root, field bindweed leaves, silver birch leaves and common bearberry leaves) for their antioxidant effects in various assays. We then formulated them into different types of food models. Based on all the research studies we have reviewed, we can draw the following conclusions:

1. White tea: The phenolic composition of plants was determined using HPLC. Study of white tea revealed various catechins including EGCG, EC, EGC and ECG. A novel method of radical scavenging activity was developed by measuring white tea against methoxy radical using ferulic acid as an antioxidant standard. This study revealed that the tea extract showed antiradical activity against methoxy radical comparable to the gallate group, EGCG and EC.
2. Yellow Gentian: The HPLC result showed the presence of the bitter constituent of secoiridoid-glycoside with gentiopicroside and sweroside in the plant extract. Using different solvents, the assays identified the phenolic content and

antioxidant activity in the yellow gentian extract. The existence of an antioxidant compound was revealed by the Post-Column On-Line Coupling $ABTS^{\bullet+}$ measurement from the absorbance peak which also was not related to the secoiridoid-glycoside compound. The effectiveness of yellow Gentian lyophilise was measured using two different food models. Oil water emulsion treated with 0.5% (w/w) lyophilise yellow Gentian was shown to act as a potent preservative against primary and secondary oxidation, whereas the combination with 0.1% (w/w) BSA showed a synergic effect and better activity in delaying lipid oxidation. Application of 2 g kg^{-1} lyophilise yellow Gentian effectively controlled lipid oxidation in high and low oxygen concentration atmospheres in meat patties. A combination of 2 g kg^{-1} lyophilised yellow Gentian with 0.5 g kg^{-1} ascorbic acid showed a synergic effect that inhibited loss of redness and reduced the oxidation rate in meat patties. Sensory analysis indicated the potential of using 2 g kg^{-1} Gentiana lutea without significantly altering the appearance and taste of the modified patties.

3. Field bindweed: The plant extract with 50% ethanol showed excellent antioxidant activity when measured by FRAP, TEAC and ORAC antioxidant activity assays. It also showed the ability to scavenge methoxy radical generated by Fenton reaction measured by EPR. The plant extract also showed a protective effect against lipid degradation and the reduction of redness in the meat model by adding 0.3% (w/w) lyophilised extract. Using a gelatin-based film coated with field bindweed resulted in a significant delay in lipid degradation in beef.
4. Common bearberry: Our study identified the positive potential of common bearberry extract in terms of antioxidant activity, scavenging ability, delay of lipid oxidation in emulsion and its use in active packaging with a gelatin film. BL showed high phenolic compound content as well as antioxidant activity in the total phenolic content and TEAC assay. The methanol extract showed scavenging ability against methoxy radicals by means of the Fenton reaction, which we assessed by EPR. Lyophilised Common bearberry at 0.1% (w/w) can be applied as an antioxidant in an o/w emulsion, which significantly inhibits the lipid oxidation during 20 days of storage. The study of gelatin-based film coated

with common bearberry extract demonstrated that the extract has the ability to significantly delay the degradation of lipids in meat patties.

5. Silver birch: We used HPLC-LCMS to detect active phenolic compounds in a silver birch extract. The phenolic compounds of silver birch include catechin, p-coumaric acid, myricetin, quercetin and naringenin. BP extract showed high phenolic content and antioxidant activity in 50% aqueous ethanol measured by FRAP, TEAC and ORAC assays. The silver birch extract showed scavenging ability towards methoxy radicals generated by the Fenton reaction, as assessed by EPR. Lyophilised BP at 0.3% (w/w) was shown to decrease the discolouration and browning of meat, as measured by the metmyoglobin assay, and significantly delayed the oxidation rate of the meat. A gelatin-based film coated with 0.1% (w/w) silver birch caused significant delay to the degradation of lipids in meat.

4.2 Recommendation for Future Research

Recommendations for future research derived from this thesis are listed below:

1. The active compounds of plant extracts should be identified using high throughput equipment such as LCMS or HPLC-LCMS. Active constituents relevant to antioxidants should also be measured using Post-Column On-Line Coupling $ABTS^{\bullet+}$ HPLC for each plant extract. The Post-Column On-Line Coupling $ABTS^{\bullet+}$ method is important to identify precisely which compound available in the extract is responsible for the antiradical activity against $ABTS^{\bullet+}$. From this suggestion, we can address the relevant compound that is responsible for the activity and isolate it for future analysis in an antimicrobial or anticancer assay.
2. The formulation of plant extracts in active film packaging should be researched. The study of the characteristics of film packaging using natural plants can be enhanced by measuring the film morphology and the controlled release of active

compounds into the system. The film strength and durability should also be improved as a way of replacing commercial plastics in the market.

3. Anti-microbial analysis should also be incorporated into the research because the oxidation rate is greatly affected by the microbial count in foods.

ANNEX

PUBLICATION AND CONFERENCES

Journal Publication

1. **Azman NAM**, Peiró S, Fajarí L, Julià L, Almajano MP. Radical scavenging of white tea and its flavonoid constituents by electron paramagnetic resonance (EPR) spectroscopy. *J Agric Food Chem*. 2014;62(25):5743-5748.

2. **Azman NAM**, Gordon MH, Skowyra M, Segovia F, Almajano MP. Use of lyophilised and powdered Gentiana lutea root in fresh beef patties stored under different atmospheres. *J Sci Food Agric*. 2014. Epub ahead of print.

3. **Azman NAM**, Segovia F, Martínez-Farré X, Gil E, Almajano MP. Screening of Antioxidant Activity of Gentian Lutea Root and Its Application in Oil-in-Water Emulsions. *Antioxidants*. 2014;3(2):455-471.

4. **Azman NAM**, Gallego MG, Juliá L, Fajari L, Almajano MP. The Effect of Convolvulus arvensis Dried Extract as a Potential Antioxidant in Food Models. *Antioxidants*. 2015;4(1):170-184.

5. **Azman NAM**, Gallego MG, Shafik H, Almajano MP. Solvent Effect on Antioxidant Activity and Total Phenolic Content of Betula Pendula roth. and Convolvulus arvensis. *International Journal of Biological, Food, Veterinary and Agricultural Engineering*. 2013;7(5):152-157.

6. Skowyra M, Falguera V, **Azman NAM,** Segovia F, Almajano MP. The Effect of Perilla frutescens Extract on the Oxidative Stability of Model Food Emulsions. *Antioxidants*. 2015;4(1):170-184.

7. Gómez FS, Sánchez SP, Iradi MGG, **Azman NAM**, Almajano MP. Avocado Seeds: Extraction Optimization and Possible Use as Antioxidant in Food. *Antioxidants*. 2014;3(2): 439-454.

Journal Under Review

1. **Azman NAM**, Almacellas J, Fajarí L, Julià L, Almajano MP. Study of Bearberry Leaves Dried Extract as Potential Antioxidant in Food Models. *Journal of the American Oil Chemists' Society*. Under Review.
2. **Azman NAM**, Gallego MG, Fajarí L, Julià L, Almajano MP. Evaluation of the *Betula Pendula Roth.* Leaves extract (BP) potential on food models. *Meat Science*. Under Review.

6.3 Conferences

1. VII CONGRESO CYTA - CIENCIA Y TECNOLOGÍA DE LOS ALIMENTOS

   Venue : Universidad de Córdoba, Spain

   Date : 12-14th June 2013

   Proceeding title:

   I. Evaluation of antioxidant activity of three plants in the Pyrenees: *Betula alba*, *Convolvulus arvensis* and *Malva Slyvestris*.
   II. Effect of solvent on the polyphenol content and antioxidant activity of *Betula Alba* and *Convolvulus Arvensis*
   III. Comparative study of the antioxidant activity of aqueous ethanol extracts from different parts of *Trifolium Alpinum*

2. ICBFE 2013 : International Conference on Biotechnology and Food Engineering
   Venue : Rome, Italy
   Date : 26-27th September 2013

   Proceeding title: Solvent Effect on Antioxidant Activity and Total Phenolic Content of *Betula alba* and *Convolvulus arvensis.*

3. 28th EFFoST Conferences : 7th International Conference on the Food Factory for the Future
   Venue :Uppsala, Sweden
   Date: 25-28th November 2014

   Proceeding title:

   I. Antioxidant activity and total phenolic content of 5 edible plants from Pyrenees.
   II. Application of *Arctostaphylos uva-ursi's* leaves extract to food systems.